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(54) Title: PROCESS FOR TRANSFORMING MONOCOTYLEDONOUS PLANTS

(57) Abstract

Novel method of transforming monocotyledonous plants, particularly cereals, by wounding and/or degrading the cells of either intact tissue of the plant that is capable of forming compact embryogenic callus or the compact embryogenic callus itself.

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## PROCESS FOR TRANSFORMING MONOCOTYLEDONOUS PLANTS

This invention relates to a rapid and efficient method for transforming monocotyledonous plants generally, especially gramineous plants, particularly corn and other major cereals. The invention particularly relates to the use of either intact tissue capable of forming compact embryogenic callus or compact embryogenic callus obtained from such tissue to obtain transgenic monocotyledonous plants.

This invention also relates to novel transgenic gramineous plants, particularly cereals, which can be obtained by the transformation method of this invention.

### Background of the Invention

In recent years, there has been a tremendous expansion of the capabilities for the genetic engineering of plants. Many transgenic dicotyledonous plant species have been obtained. However, many species of plants, especially those belonging to the Monocotyledonae and particularly the Gramineae, including economically important species such as corn, wheat and rice, have proved to be very recalcitrant to stable genetic transformation.

Difficulties have been encountered in achieving both: a) integrative transformation of monocot plant cells with DNA (i.e., the stable insertion of DNA into the nuclear genome of the monocot plant cells) and b) regeneration from transformed cells of phenotypically normal monocot plants, such as phenotypically normal, fertile adult monocot plants. It has been suggested that such difficulties have been predominantly due to the nonavailability of monocot cells that are competent with respect to: 1) DNA uptake, 2) integrative

transformation with the taken-up DNA, and 3) regeneration of phenotypically normal, monocot plants from the transformed cells (Potrykus (1990) Bio/Technology 9:535). In general, direct gene transfer into protoplasts (using polyethyleneglycol treatment and/or electroporation) has seemed to have the best potential for success. Protoplasts for use in such direct gene transfer methods have most often been obtained from embryogenic cell suspension cultures (Lazzeri and Lörz (1988) Advances in Cell Culture, Vol.6, Academic press, p. 291; Ozias-Akins and Lörz (1984) Trends in Biotechnology 2:119). However, the success of such methods has been limited due to the fact that regeneration of phenotypically normal plants from protoplasts has been difficult to achieve for most genotypes.

Recently, success has been reported in the transformation of, and regeneration of phenotypically normal plants from, certain lines of rice (Shimamoto et al (1989) Nature 338:274; Datta et al (1990) Bio/Technology 8:736; and Hayashimoto et al (1990) Plant Physiol. 93:857) and corn (Gordon-Kamm et al (1990) Bio/Technology 2:603; Fromm et al (1990) Bio/Technology 8:833; Gould et al (1991) Plant Physiology 95:426; and PCT publications WO91/02071 and WO89/12102). However, it is not clear from such reports that their processes of transformation and regeneration are applicable to monocots generally, particularly gramineous plants, quite particularly cereals.

## Summary of the Invention

This invention provides a novel method for efficiently and reproducibly transforming the genome of a monocotyledonous plant, particularly a gramineous plant such as a major cereal (e.g., corn, wheat, rice,

rye, etc). This method comprises the transformation with DNA of cells of either: a) an intact tissue of the monocotyledonous plant, which tissue is capable of forming compact embryogenic callus or b) a compact embryogenic callus, particularly its embryogenic sectors, obtained from such intact tissue, such cells being competent with respect to: 1) uptake of the DNA, 2) integrative transformation of the plant genome, preferably its nuclear genome, with the DNA and 3) regeneration of the phenotypically normal plant (e.g., phenotypically normal, fertile adult plant) from the cells following the transformation of their genome. Such competent cells are preferably obtained by wounding and/or degrading the intact tissue or the compact embryogenic callus of the plant, for example by: a) cutting either the intact tissue and the cells thereof or the compact embryogenic callus and the cells thereof obtained from such intact tissue; and/or b) depending upon the nature of the intact tissue or the compact embryogenic callus, treating the intact tissue or the compact embryogenic callus with an enzyme to degrade the cell walls of the intact tissue or compact embryogenic callus.

The resulting wounded and/or degraded, intact tissue or compact embryogenic callus, containing the competent cells of this invention, can be transformed, preferably by direct gene transfer, such as by means of electroporation, with one or more DNA fragments (e.g., foreign DNA fragments), preferably linear DNA fragments. Preferably, at least one of the DNA fragments contains a gene which can serve as a selectable or a screenable marker, preferably a selectable marker, for transformed plant cells. Such a marker DNA fragment can be located on the same DNA

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fragment or on a separate DNA fragment as another gene
or other gene(s) of interest.

The transformed cells can be separated in a
conventional manner from non-transformed cells by
culturing on a selective medium, preferably for a
prolonged time, and the transformed cells, thus
selected, can be regenerated in a conventional manner
into phenotypically normal plants (e.g., mature plants)
which possess the gene(s) of interest stably integrated
in their genomes, particularly their nuclear genomes.

This invention also provides: novel competent
cells of monocot plants, especially gramineous plants,
particularly cereal plants, the genomes of which have
been stably transformed with one or more DNA fragments;
cell cultures consisting of such transformed cells;
phenotypically normal plants (e.g., phenotypically
normal, fertile plants) regenerated from such
transformed cells; and seeds of such transformed
plants. Among such transformed cells, cell cultures,
plants and seeds are those transformed with a DNA
fragment containing a gene that encodes a protein
capable of killing or disabling a plant cell in which
the protein is expressed and that is under the control
of the tapetum-specific PTA29 promoter whereby the
plants are male sterile. The transformed gramineous
plants of this invention, particularly transformed corn
and rice, are characterized by their being from plant
lines, from which it is practically impossible with
conventional techniques to regenerate the transformed
plants, as phenotypically normal plants, from
transformed embryogenic suspension cultures or from
transformed protoplasts, particularly where for every
10,000 untransformed protoplasts of such plant lines,
no more than about 500, especially no more than about
100, particularly no more than about 10, quite

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particularly no more than about 1, phenotypically normal plant(s) can be regenerated.


Brief Description of the Drawings

Figure 1: NPTII gel assays of Example 2 of five corn transformants obtained by electroporation of immature zygotic embryos.

Figure 2: Southern blots of Example 2 of genomic DNA of one of the corn transformants of Example 1 (H99-M148-1), using the sequence listed as Seq. Id. 2 as a probe. Lengths of standard fragments are indicated. The origin is indicated by O.

  Lanes:  1 : PstI digested DNA of phage lambda + HindIII digested pTTM1 (positive control - probe should hybridizes to 2824 bp pTTM1 fragment)

          2 : BglII digested genomic DNA

          3 : EcoRI digested genomic DNA

          4 : EcoRV digested genomic DNA

          5 : HindIII digested genomic DNA

          6 : BamHI digested genomic DNA

          7 : PvuI digested genomic DNA

          8 : PvuII digested genomic DNA

          9 : PstI digested genomic DNA

          10: EcoRI digested plant genomic DNA of untransformed H99 plant (negative control)

Figure 3: NptII gel assays of Example 4 of seven transformants obtained by electroporation of compact embryogenic callus fragments derived from immature zygotic embryos.

Figure 4: Southern blots of Example 4 of genomic DNA of one of the corn transformants of Example 3

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(Pa91-M146-2) using the sequence listed as
Seq. Id. 2 as a probe. Lengths of standard
fragments are indicated. The origin is
indicated by O.

Lanes:  1 : PstI digested DNA of phage lambda
            + HindIII digested pTTM1 (positive
            control - probe should hybridizes to
            2824 bp pTTM1 fragment)
        2 : BglII digested genomic DNA
        3 : EcoRI digested genomic DNA
        4 : EcoRV digested genomic DNA
        5 : HindIII digested genomic DNA
        6 : BamHI digested genomic DNA
        7 : PvuI digested genomic DNA
        8 : PvuII digested genomic DNA
        9 : PstI digested genomic DNA
       10 : EcoRI digested plant genomic DNA
            (negative control)

SEQUENCE LISTING

Seq. Id. No. 1 :  sequence of pDE108

Seq. Id. No. 2 :  sequence of probe used to detect
                  chimaeric neo gene in Southern
                  hybridizations

Seq. Id. No. 3 :  sequence of a DNA fragment of plasmid
                  pTTM8 used in the construction of
                  plasmids pVE107 and pVE108 and
                  comprising the promoter from the TA29
                  gene of tobacco and the barnase gene

Seq. Id. No. 4 :  sequence of pDE110

Detailed Description of the Invention

In monocots, embryogenic callus can be of two
distinct and well known types (see: Vasil (1988)
Bio/Technology 6:397; Armstrong and Green (1988) Crop

Sci. 28:363). One type of embryogenic callus can best
be described as compact and/or nodular and can often be
considered as organized. Such callus, termed herein
"compact embryogenic callus", is used in accordance
with this invention. The other and generally less
frequently occurring type of embryogenic callus can
best be described as soft, friable and highly
embryogenic, and such callus, termed herein "friable
embryogenic callus", generally grows faster than the
compact embryogenic callus. From either type of callus,
phenotypically normal plants can be regenerated, and in
both types of callus, somatic embryos are present in
different stages of development. The appearance and
final morphology of the two types of callus can differ
in different monocot species, particularly in different
cereal species. Nevertheless, the two types of callus
can be readily distinguished from one another by
persons skilled in the art of forming and manipulating
tissue cultures of different monocot species.

In corn, compact embryogenic callus and friable
embryogenic callus are more familiarly known as type I
callus and type II callus, respectively. Various
distinguishing features in the structure and properties
of type I and type II corn calli are described in
publications, such as: Armstrong and Phillips (1988)
Crop Sci. 28:363; Springer et al (1979) Protoplasma
101:269; Fransz (1988) "Cytodifferentiation during
callus initiation and somatic embryogenesis in Zea mays
L.", Ph.D. Thesis, University of Wageningen, The
Netherlands; Ozias-Akins et al (1982) Protoplasma
110:95; Novak et al (1983) Maydica 28:381; Ho et al
(1983) Protoplasma 118:169; Green et al (1975) Crop
Sci. 15:417; Freeling et al (1976) Maydica 21:97; Lu et
al (1982) Theor. Appl. Genet. 62:109; Vasil et al
(1985) Protoplasma 127:1; Dunstan et al (1978)

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Protoplasma 97:251; Vasil et al (1982) Bot. Gaz. 143:454; Green (1983) In: Basic biology of new developments in biotechnology, Hollaender et al (eds) Plenum Press, New York, pp. 195-209; Vasil et al (1984) Am. J. Bot. 71:158; and Kamo et al (1985) Bot. Gaz. 146:327.

Type I corn callus is essentially white, pale white or yellowish and compact in appearance, often has a nodular surface, and represents the generation and propagation of an organized set of tissues which is reflected in its nodular appearance. It is characterized by a high degree of cellular association and differentiation and by various structures, such as roots, leafy structures and vascular elements. Somatic embryos can generally be recognized. The origin of regenerated shoots is not always obvious and can apparently occur by both somatic embryogenesis and organogenesis. During somatic embryo development, embryoids can fuse and give rise to hard, white callus or can develop into secondary somatic embryos.

Type II corn callus is essentially soft, friable, white or pale-yellow, somewhat transparent in appearance and highly embryogenic. It grows rapidly and contains no vascular elements. Type II callus differs from non-embryogenic friable callus in containing numerous smooth and globular embryoids that may possess a suspensor-like structure by which the embryoids are attached to the callus. The embryoids can develop into well-organized somatic embryos.

Approximately the same distinguishing features, that are found in the two types of corn calli, can be used to distinguish between the compact embryogenic callus and the friable embyogenic callus of other monocot species, particularly cereal species such as rice (Kyozuka et al (1988) Theor. Appl. Genet. 76:887),

wheat (Redway et al (1990) Theor. Appl. Genet. 76:609;
Redway et al (1990) Plant Cell Reports 8:714), and
barley.

From monocotyledonous plants generally, the
compact embryogenic callus of this invention can be
obtained by in vitro culture of explant sources such as
immature zygotic embryos, mature seeds, leaf bases,
anthers, microspores, young inflorescences, etc. In
corn, the type I callus is most efficiently generated
from immature zygotic embryos. The compact embryogenic
callus can be induced from the appropriate explants and
maintained in culture according to well-established
methods (see Hodges et al (1986) Bio/Technology 4:219).
During maintenance of the callus culture, care has to
be taken to select and subculture only the embryogenic
sectors of the calli in which are the embryogenic
cells. Such cells can generally be characterized as
small, tightly packed, thin-walled, richly cytoplasmic,
highly basophilic cells containing many small vacuoles,
lipid droplets and starch grains (Vasil (1988) supra).
The most convenient way to remove, from a plant,
tissues that are known to be capable of forming the
compact embryogenic callus is by means of dissection.

The competent cells of this invention can be
obtained directly from a monocotyledonous plant by
cutting from the plant, in a conventional manner,
intact tissue that is capable of forming compact
embryogenic callus. The cells of such wounded intact
tissue can then be stably transformed. However, it is
preferred that such wounded intact tissue be cut into
smaller fragments to wound further such tissue and
provide more competent cells for transformation. The
average maximum dimension of the tissue fragments is
preferably 0.1 to 5 mm long, particularly 1 to 2.5 mm
long, more particularly 1.25 to 1.75 mm long. In this

respect, the wounded intact tissue of this invention can be any piece of tissue that is cut from the plant or any fragments thereof (e.g., cut pieces). Thus, the term "intact tissue" should be understood as referring to aggregates of monocot plant cells that are obtained from a naturally occurring plant part, without a tissue-culturing stage in between.

It is believed that the mechanical disruption or wounding of the intact tissue and its individual cells, by cutting the intact tissue from the plant and possibly further cutting it so as to disrupt or wound it further, is generally sufficient to generate the competent cells of this invention. In this regard, the terms "mechanical disruption" and "wounding" are intended to encompass the significant damaging of the cell wall of one or more cells of the intact tissue in order to expose the cell(s) and render the cell(s) open to insertion of a DNA fragment in accordance with this invention. Thus, "mechanical disruption" or "wounding" in accordance with this invention is not limited to cutting the cell wall but includes other methods of physically removing one or more portions of the cell wall or rendering the cell wall discontinuous in one or more places, such as by abrading, squeezing or striking the cell wall.

However, the mechanical disruption or wounding of the intact tissue in accordance with this invention can be supplemented or even replaced by a treatment of the intact tissue with an enzyme or enzyme mixture to degrade the plant cell walls, especially when the intact tissue is relatively large. The enzyme treatment can be carried out in a conventional manner. Preferably, the enzyme is applied to the intact tissue primarily to generate pores in its cell walls. It is therefore preferred that the enzyme treatment be

relatively short (e.g., from 1 to 10 minutes depending
upon the nature and the consistency of the intact
tissue) so as not to cause a complete disruption of the
tissue. Depending upon the type of plant, various
enzymes or enzyme solutions can be used such as those
listed by Powell and Chapman (1985) "Plant Cell
Culture, A Practical Approach", R.A. Dixon ed., Chapter
3.

When the intact tissue, obtainable from the plant,
is too small to be wounded (e.g., cut) or wounded
intact tissue is too small to be further wounded (e.g.,
cut into smaller pieces), the enzyme treatment can be
used to generate additional competent cells. Such an
enzyme treatment can also be particularly useful, by
itself, for forming competent cells of this invention
in embryos, particularly in immature zygotic embryos
isolated from developing seeds and in mature zygotic
embryos isolated from mature (e.g., dry) seeds of, for
example, corn. Embryos are generally not cut to remove
them from seeds and generally cannot be cut into
significantly smaller fragments without destroying
their ability to generate compact embryogenic callus.
Immature embryos are particularly important in corn as
they are the only convenient and reliable source of
compact embryogenic callus. In rice and other monocots,
mature embryos can also be used. In this regard, for
plants such as corn, it is preferred that the intact
tissue (e.g., immature corn embryos) have a maximum
length of about 0.5 to 2 mm, preferably 0.5 to 1.5 mm,
even though smaller lengths of 0.5 to 1 mm can be used.

In accordance with this invention, the intact
tissue is also preferably subjected to a period of, for
example, about 15 minutes or more, preferably about 30
minutes to about 5 hours, particularly 2 to 3 hours, of
preplasmolysis which involves placing the tissue in a

conventional hypertonic solution, such as the electroporation buffer discussed below. The purpose of this preplasmolysis treatment is to separate at least partly, in the cells of the intact tissue, their protoplasts, preferably all or at least part of their cell membranes, from their cell walls. Such preplasmolysis is preferably carried out after any wounding of the intact tissue but before any enzyme treatment of the intact tissue. When the intact tissue has already been degraded by an enzyme treatment, it is preferred that any subsequent preplasmolysis be only for a brief period, and after the enzyme treatment of immature embryos of corn, as discussed above, it is preferred that such preplasmolysis not be carried out at all.

The competent cells of this invention can also be obtained by: culturing *in vitro* the intact tissue of this invention to produce compact embryogenic callus; and then cutting the callus into smaller fragments. The resulting callus fragments should comprise, wholly or at least in part, the embryogenic sectors or parts of the callus. The callus fragments also preferably have an average maximum length of 0.5 to 2.5 mm, particularly 1 to 2 mm, more particularly 1.25 to 1.75 mm, and preferably have a minimum length of about 0.1 mm. To obtain sufficient amounts of compact embryogenic callus, it is preferred to propagate the primary callus, as obtained from tissue explants, for at least one month and to subculture the embryogenic sectors of such primary callus at least once during this period. It is believed that the mechanical disruption or wounding of the embryogenic sectors of the compact embryogenic callus and their cells by, for example, cutting them is generally sufficient to generate the competent cells of this invention. However, the

mechanical disruption of the callus may be supplemented
or replaced by an enzyme treatment to degrade the
callus cell walls, especially when the compact
embryogenic callus fragments remain relatively large.
This enzyme treatment can be carried out in a
conventional manner. The enzyme treatment preferably
serves primarily to generate pores in the cell walls of
the cells of the callus fragments, and it is therefore
recommended that the enzyme treatment be relatively
short, preferably from 1 to 10 minutes depending upon
the consistency of the callus fragments, so as not to
cause a complete disruption of the tissues. Depending
upon the monocot plant, various enzymes or enzyme
solutions can be used such as those listed by Powell
and Chapman (1985) supra. Preferably, the compact
embryogenic callus fragments are also subjected to a
period (e.g., 2 to 3 hours) of preplasmolysis, as
discussed above.

The wounded and/or degraded, intact tissue or
compact embryogenic callus fragments, particularly
their embryogenic sectors, obtained as described above,
are then brought into contact with one or more DNA
fragments containing gene(s) of interest in order to
transform their competent monocot plant cells of this
invention. It is preferred that at least one of the
genes of interest be adapted to serve as a selectable
marker in the resulting transformed monocot plant
cells. It is believed that direct gene transfer,
particularly electroporation, provides optimal
transformation efficiency. However, other known DNA
transfer techniques can be used such as direct gene
transfer using polyethyleneglycol, bombardment with
DNA-coated microprojectiles (i.e., biolistic
transformation using, for example, a particle gun), and
Agrobacterium-mediated transformation.

The compact embryogenic callus, used in carrying out the plant transformation method of this invention, can have certain characteristics of a friable embryogenic callus. In this regard, a compact embryogenic callus or a friable embryogenic callus can change or be caused to change into a type of callus that has some of the characteristics of compact embryogenic callus as well as some characteristics of friable embryogenic callus. As a result, such an intermediate type of callus and embryogenic portions thereof can sometimes be transformed in accordance with this invention. Indeed, somatic embryos that develop on such an intermediate type of callus, as well as on friable embryogenic callus, can be isolated and can be wounded and/or degraded and then transformed as described above. Thus, in carrying out the method of this invention, such somatic embryos obtained from an intermediate type callus or a friable embryogenic callus can be regarded as equivalent to immature or mature zygotic embryos obtained from developing or mature seeds, particularly when electroporation is used as the means for transforming cells of the somatic embryos.

In accordance with this invention, electroporation can be carried out in a conventional manner. In this regard, the wounded and/or degraded intact tissue or callus fragments, particularly meristematic or embryogenic sections thereof, quite particularly embryogenic sections thereof, can be transferred to a cuvette suitable for use in an electroporation apparatus (e.g., as described by Dekeyser et al (1990) The Plant Cell 2:591). Preferably, about 10 to 500 mg, particularly about 50 to 200 mg, most particularly about 100 to 150 mg, of intact tissue or callus fragments per 200 $\mu$l of electroporation buffer are

transferred to the cuvette. For cereals, such as corn, (where it is preferred to use intact enzyme-treated immature embryos), it is preferred that about 10 to 500 embryos, particularly about 50 to 150 embryos, more particularly about 75 to 125 embryos, in 200 $\mu$l of electroporation buffer are transferred to the cuvette. The DNA is then added to the cuvette, and the electroporation is carried out. Preferably, the DNA is coincubated (e.g., for about 1 hour) with the intact tissue or callus fragments prior to electroporation. It is believed that best results can be obtained with linear, rather than circular, DNA of relatively small size, preferably smaller than about 20 kb, especially smaller than 15 kb, particularly smaller than 10 kb, quite particularly smaller than 6 kb (e.g., down to about 2-3 kb). In this regard, multiple linear DNA fragments of different composition can be used to transform the competent cells of this invention with multiple genes of interests. Preferably, about 5 to 30 $\mu$g, particularly about 10-25 $\mu$g, quite particularly about 20 $\mu$g, of DNA is added to the cuvette containing the intact tissue or callus fragments. Particular electroporation conditions are not believed to be critical, and good results can be obtained, for example, with a discharge of one pulse with a field strength of 375 V/cm from a 900 $\mu$F capacitor using an electroporation buffer containing 150 mM NaCl or 80 mM KCl (Dekeyser et al (1990) supra).

When the transformation (e.g., by electroporation) is completed, the intact tissue or callus fragments, containing the transformed monocot cells, are transferred to a suitable culture medium, preferably a selective medium when the transformed cells contain a selectable marker. This transfer should be as soon as possible after, preferably immediately after, the

transformation event and especially within one to three days after the transformation event. Preferably, the intact tissue or callus fragments transformed with a selectable marker are cultured using conventional culture conditions and culture media (see, e.g., references in Vasil (1988) supra) supplemented with a selective agent. The selection of the selective agent will depend on the selectable marker used in the DNA fragments to transform the cells of the intact tissue or callus fragments, as discussed below. The concentration of the selective agent should provide a very high selective pressure on the transformed cells so that only stable transformants, in which the DNA fragments containing the selectable marker are integrated, preferably fully integrated, in the genome of the cells, survive and can be isolated. Although such transformed intact tissue or callus fragments can be cultured for a few days on non-selective medium, it is preferred that they be transferred to selective medium as soon as possible and maintained for a prolonged period (e.g., as long as six months), preferably at least one month, especially two to three months, to produce significant amounts of transformed morphogenic callus, such as transformed compact embryogenic callus, which can be used to regenerate a phenotypically normal plant. It is also preferred that the hypertonicity of the medium be maintained for a limited time (e.g., up to two to three weeks), for instance by supplementing the medium with mannitol.

In accordance with this invention, any DNA fragment can be integrated in the genome, particularly the nuclear genome, of a monocotyledonous plant. Generally, the DNA fragment contains a foreign or endogenous gene or other DNA sequence which is functional in the transformed plant cells and confers

an additional property to such cells and to plants
regenerated from the cells. To this end, the DNA
fragment preferably comprises one or more chimaeric
genes which contain the following operably linked DNA
sequences: 1) a promoter sequence capable of directing
expression of a coding sequence in the plant cell (a
"promoter"); 2) a sequence (a "coding sequence") coding
for a protein with a specific activity within the plant
cell (a "protein of interest"): and 3) suitable 3'
transcription regulation signals. In order to obtain
the required functionality of the protein, it may also
be necessary that the protein be targeted to one or
more particular compartments of the plant cell, such as
the       cytosol,     mitochondria,    chloroplasts     or
endoplasmatic reticulum. For targeting to the cytosol,
the chimaeric gene, as described above, can be used as
such. However for targeting to the other compartments,
it is required that there be an additional sequence (a
"targeting sequence") between the DNA fragments 1) and
2) of the chimaeric gene. If required, the chimaeric
gene   can   also   contain   transcriptional   and/or
translational enhancers, and the codon usage of the DNA
sequences can be optimized for expression in plant
cells.

     Chimaeric genes in accordance with this invention
can   be   constructed   according   to   well-established
principles and techniques. In this regard, the various
DNA sequences should be linked so that translation is
initiated at the initiation codon of the coding
sequence of the protein (or of the targeting sequence
when it is present).

     It is believed that the  various constitutive and
organ- and tissue-specific promoters that are presently
used to direct expression of genes in transformed
dicotyledonous plants will also be suitable for use in

transformed monocots of this invention. In this regard, particular plant cells can be transformed with a chimaeric gene comprising: a coding sequence encoding a protein of interest; and upstream (i.e., 5') thereof, either a foreign or an endogenous promoter suitable for expression of the coding sequence. Suitable foreign constitutive promoters include: the promoter of the Cauliflower Mosaic Virus ("CaMV") isolates CM1841 (Gardner et al (1981) Nucl. Acids. Res. 9:2871) and CabbB-S (Franck et al (1980) Cell, 21:285) (the "35S promoter") which directs constitutive expression of heterologous genes (Odell et al (1983) Nature 313:810); a related promoter (the "35S3 promoter") which can be isolated from the CaMV isolate CabbB-JI (Hull and Howell (1978) Virology 86:482) and which differs from the 35S promoter in its sequence (the sequence of the 35S3 promoter is disclosed in European patent publication ("EP") 359617) and in its greater activity in transgenic plants (Harpster et al (1988) Mol. Gen. Genet. 212:182); and the TR1' and the TR2' promoters which drive the expression of the 1' and 2' genes, respectively, of the T-DNA of <u>Agrobacterium</u> (Velten et al (1984) EMBO J. 3:2723) and are wound-induced promoters. Suitable organ-specific, tissue-specific and/or inducible foreign promoters are also known (see, e.g., references cited in Kuhlemeier et al (1987) Ann. Rev. Plant Physiol. 38:221) such as the promoters of the small subunit genes (such as the 1A gene) of 1,5-ribulose bisphosphate carboxylase of <u>Arabidopsis thaliana</u> (the "ssu" promoter) which are light inducible promoters (Krebbers et al (1988) Plant Mol. Biol. 11:745) active only in photosynthetic tissue; the anther-specific promoters disclosed in EP 344029; and the seed-specific promoters of, for example, <u>Arabidopsis thaliana</u> (Krebbers et al (1988) Plant

Physiol. 87:859). Promoters of particular usefulness for transforming monocots to render them male-sterile, as described in EP 344029, are the tapetum-specific promoters PTA29, PTA26 and PTA13, particularly PTA29, of EP 344029.

Likewise, it is believed that known 3' transcription regulation sequences and polyadenylation signals used in transformed dicotyledonous plants can be used in transformed monocots of this invention. Such 3' transcription regulation signals can be provided downstream (i.e. 3') of the coding sequence. In this regard, a particular plant cell can be transformed with a chimaeric gene containing either foreign or endogenous transcription termination and polyadenylation signals suitable for obtaining expression of the chimaeric gene. For example, the foreign 3' untranslated ends of genes, such as gene 7 (Velten and Schell (1985) Nucl. Acids Res. 13:6998), the octopine synthase gene (Gielen et al (1983) EMBO J. 3:835) and the nopaline synthase gene of the T-DNA region of Agrobacterium tumefaciens Ti-plasmid can be used.

For construction of a chimaeric gene which can be expressed in a transformed plant cell, preferably in its cytoplasm followed by translocation of its protein of interest to the cell's mitochondria, chloroplasts and/or lumen of the endoplasmatic reticulum, suitable targeting sequences are known. Selection of such targeting sequences is not believed to be critical, and a particular plant cell can be transformed with a chimaeric gene containing either a foreign or endogenous targeting sequence encoding a targeting peptide which will provide translocation of the expression product of the gene. By "targeting peptide" is meant a polypeptide fragment which is normally

associated, in an eucaryotic cell, with a chloroplast
or mitochondrial protein or subunit of the protein or
with a protein translocated to the endoplasmatic
reticulum and which is produced in a cell as part of
precursor protein encoded by the nuclear DNA of the
cell. The targeting peptide is responsible for the
translocation process of the nuclear-encoded
chloroplast or mitochondrial protein or subunit into
the chloroplast or the mitochondria or the lumen of the
endoplasmatic reticulum. During the translocation
process, the targeting peptide is separated or
proteolytically removed from the protein or subunit. A
targeting sequence can be provided in the chimaeric
gene to express a targeting peptide which can
translocate an expressed protein of interest within a
transformed plant cell as generally described in
European patent applications ("EPA") 85402596.2 and
88402222.9. A suitable targeting peptide for transport
into chloroplasts is the transit peptide of the small
subunit of the enzyme 1,5-ribulose bisphosphate
carboxylase (Krebbers et al (1988) Plant Mol. Biol.
11:745; EPA 85402596.2), but other chloroplast transit
peptides, such as those listed by Watson (1984) Nucl.
Acids Res. 12:5145 and Von Heijne et al (1991) Plant
Mol. Biol. Rep. 9:104, can also be used. Suitable
mitochondrial targeting peptides are the mitochondrial
transit peptides as described by Schatz (1987) Eur. J.
Biochem. 165:1 and listed by Watson (1984) supra.
Suitable targeting peptides that can translocate a
protein of interest to the lumen of the endoplasmatic
reticulum of a plant cell are, for instance, the signal
peptides described by Von Heijne (1988) Biochem.
Biophys. Acta 947:307 and listed by Watson (1984)
supra.

Coding sequences that can be used for the
production of transgenic dicotyledonous plants are well
known (see, for example, the coding sequences listed in
Weising et al (1988) Annual Rev. Genet. 22:421), and it
is believed that such coding sequences can be put to
equally good use in transformed monocotyledonous plants
in accordance with this invention. In this respect, the
coding sequences can be either foreign or endogenous to
the plants and can, for example, code for proteins
which: are toxic to insect species, thus protecting the
plants against insect attack (EP 193259, EP 305275 and
EP 358557); protect the plants against stress
conditions (EP 359617); confer on the plants a
resistance or tolerance to specific herbicides (EP
242236); kill or disable plant cells in which the
proteins are expressed so that, when the coding
sequences are under the control of a male or female
organ-specific promoter, the proteins can render the
plants respectively male sterile (EP 344029) or female
sterile (EP 412006); can be extracted from the plants
or selected plant organs and optionally be further
processed so that the plants can be used as sources of
economically important peptides or proteins (EP
319353); or are enriched in nutritionally important
amino acids so that transformed plants or their organs,
in which the proteins are expressed, can be used as
food with enhanced nutritional value for animals or
humans (EP 318341).

Coding sequences of particular usefulness for
transforming monocots to render them insect-resistant
are the genes isolated from <u>Bacillus</u> <u>thuringiensis</u>
("Bt") strains and truncated portions thereof that code
for insecticidal crystal proteins and their
insecticidal polypeptide toxins (for a review, see:
Höfte and Whiteley (1989) Microbiol. Rev. 53:242). The

following Bt genes are believed to be particularly important for insect control in cereals (e.g., corn, rice, wheat and barley): the CryIAb gene (EP 193259) and CryIAc gene for control of Helicoverpa species (e.g., H. zea and H. armigera); the CryIAb gene and the CryIb gene (EP 358557) for control of Ostrinia species (e.g., O. nubilalis) in corn; the CryIAc gene for the control of Agrotis species in corn and wheat; and the CryID and CryIE genes (EP 358557) for the control of Spodoptera species (e.g., S. frugiperda) in corn. To achieve sufficient expression of such genes in tissues of transgenic plants, it is preferred that the genes be modified as described in PCT application PCT/EP 91/00733 (PCT publication WO 91/16432).

Selectable markers in accordance with this invention are chimaeric genes in which the coding sequences encode proteins which confer on the plant cells, in which they are expressed, resistance to a selectable agent such as an antibiotic and/or herbicide. Screenable markers in accordance with this invention are chimaeric genes in which the coding sequences encode proteins which confer of the plant cells, in which they are expressed, a different appearance, such as a different color, making plants transformed with the screenable marker separable manually. The selection of a selectable or screenable marker, preferably a selectable marker, for transforming a monocotyledonous plant in accordance with this invention is not believed to be critical, and it is believed that conventional selectable and screenable markers can be used (see, for example, the markers listed in Weising et al (1988) supra). Examples of suitable coding sequences for selectable markers are: the neo gene (Beck et al (1982) Gene 19:327) that codes for the enzyme neomycin phosphotransferase which

confers resistance to the antibiotic kanamycin; the hyg
gene (Gritz and Davies (1983) Gene 25:179) that codes
for the enzyme hygromycine phosphotransferase which
confers resistance to the antibiotic hygromycin; and
the bar gene (EP 242236) that codes for
phosphinothricin acetyl transferase which confers
resistance to the herbicide phosphinothricin. In using
a selectable marker gene coding for a protein that
confers tolerance or resistance to a herbicide or other
selective agent that acts on chloroplast metabolism,
such as the bar gene, it is preferred that the marker
gene be part of a chimaeric gene together with a
chloroplast targeting sequence as described above.
Examples of suitable coding sequences for screenable
markers are the gus gene (Jefferson et al (1986) Proc.
Natl. Acad. Sci. USA 6:3901) encoding beta-
glucuronidase and the luciferase gene (Ow et al (1986)
Science 234:856).

As discussed above, the selection pressure,
provided by the presence of a selectable agent, should
preferably be rather high during culturing of
transformed plant cells of this invention containing
selectable markers. For example, when the neo gene is
used as a selectable marker, kanamycin should be used
in concentrations of at least about 100-200 mg per
liter, preferably at least about 200 mg per liter, in
the culture medium. Such high selection pressure should
also be maintained for a prolonged time, for example,
two to four months. It is believed, however, that
particular selection pressures and durations are not
critical and that the choice of selection pressures and
their durations can be made in a conventional manner.
However when the bar gene is used as a selectable
marker gene, phosphinothricin (PPT) is preferably used

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in concentrations of 0.5 to 50, particularly 2 to 20, mg per liter of the culture medium.

Morphogenic sectors, preferably embryogenic sectors, of morphogenic callus, preferably compact embryogenic callus, produced in a culture of transformed cells of wounded and/or degraded intact tissue or wounded and/or degraded embryogenic sectors of compact embryogenic callus of this invention, can then be regenerated into phenotypically normal (e.g., mature and fertile) plants in a conventional manner (see, for example, references in Vasil (1988) supra and Lazzeri and Lörz (1988) supra). The regenerated plants, thus obtained, will be transgenic and will at least possess the selectable or screenable marker, preferably the selectable marker, stably integrated into their nuclear genome. The presence and expression of other genes of interest can then be evaluated in a conventional manner, such as by means of Southern blotting and/or by the polymerase chain reaction (Sambrook et al (1990) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory).

For the purposes of this invention, a phenotypically normal plant as produced by the transformation and regeneration procedures of this invention should be understood as at least one plant that does not differ substantially from an untransformed plant of the same line in any of its phenotypic characteristics except in those characteristics that are added or changed due to the expression of the DNA fragments introduced in the plant's genome during transformation. Of course, any procedure for transforming plants usually produces a number of transgenic plants that display a variety of phenotypes, only some of which are phenotypically normal as defined above.

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The method of this invention can be applied to all monocotyledonous plant species from which compact morphogenic callus, such as compact embryogenic callus, can be obtained during in vitro culture of explants derived from various explant sources such as immature and mature zygotic embryos, leaf bases, young inflorescences, etc. The method will be especially useful for the transformation of economically important gramineous crops, particularly the major cereals, such as corn, wheat, rice, oats, barley, sorghum, rye and millet. The resulting transgenic plants of this invention can be used to create, in a rapid and efficient manner, novel lines and/or cultivars of high agronomic value. In this regard, transgenic plants can be created in accordance with this invention that can be used as pollinator plants, for example, as female-sterile pollinator plants for the production of hybrid seed as disclosed in EP 412006 (which is incorporated herein by reference).

This invention provides a rapid, efficient and reproducible method for transforming monocotyledonous plants, using intact tissue or compact embryogenic callus to produce cultures of transformed morphogenic callus, preferably compact embryogenic callus. This is surprising as neither intact tissue nor compact embryogenic callus has generally been regarded as a suitable starting material for obtaining stable transformants (see Vasil (1990) Bio/Technology 8:797). The use of intact tissue or compact embryogenic callus in accordance with this invention is a distinct improvement on existing monocot transformation methods 7which have required the use of friable embryogenic callus, embryogenic cell suspension cultures and/or protoplasts that are competent for: 1) DNA uptake, 2) integrative transformation and 3) efficient and

reproducible, monocotyledonous plant regeneration. Such requirements of competence have, up to now, limited stable transformations of monocots to plant lines with very specific tissue culture properties. In corn for example, only certain lines, such as the inbred line A188, have had the capacity to form enough type II callus (i.e., to form type II callus at frequencies higher than 10%, up to, for example, 80% or more), from which competent suspension cultures and/or protoplasts could be obtained at appreciable frequencies. However, all such corn lines have been of low agronomic value, so that transformations of economically valuable corn lines have only been possible by laborious breeding programs in which appropriate tissue culture properties have been transferred to the valuable corn lines from the transformable low value lines.

Because the method of this invention requires only a relatively short period of _in vitro_ culture, the method is far less time and labor consuming than previous methods. The short tissue culture period also ensures that the occurrence of somaclonal variation is reduced.

The method of this invention provides novel, phenotypically normal (e.g., fertile), transgenic monocotyledonous plants, particularly gramineous plants, quite particularly cereals, most particularly corn and rice, which are transformed with at least one (e.g., foreign) gene of interest, stably integrated into their nuclear genome. The method is believed to be independent of the genotype of the plant, being transformed, and capable of transforming cells of any plant, from which compact embryogenic callus can be obtained from at least one of its tissues. This makes it possible to transform the majority of monocot species and a substantial number of lines within each

species. Moreover, the capacity to form compact embryogenic tissue can be transferred, by means of classical breeding programs, from one line that posesses such capacity to another line that does not.

The novel transgenic monocot plants of this invention regenerated from transformed morphogenic callus, particularly transformed compact embryogenic callus, are characterized by the fact that from such monocots, using conventional culture conditions as described, for example, in Datta et al (1990) supra, Shimamoto et al (1989) supra, Hayashimoto et al (1990) supra, Gordon-Kamm et al (1990) supra, and Fromm et al (1990) supra, it is practically impossible to obtain embryogenic suspension cultures and/or protoplasts or it is practically impossible to obtain embryogenic suspension cultures and/or protoplasts which have sufficient capability of being stably transformed and then regenerated as phenotypically normal (e.g., fertile), transgenic plants. In regard to this second type of impossibility, it is not believed practical to obtain embryogenic suspension cultures or protoplasts of such monocots that: 1) have a high probability of being regenerable into phenotypically normal plants; 2) have a high probability of being competent with respect to DNA uptake and integrative transformation of the so taken-up DNA; and 3) when so transformed, have a high probability of being regenerable into phenotypically normal, transgenic plants.

In particular, this invention provides novel transgenic rice plants of rice lines, from which embryogenic suspension cultures (when obtainable) can generally be obtained, for example, according to the procedures described by Li et al (1990) Plant Mol. Biol. Report. 8:276, Datta et al (1990) Plant Sci.

67:83, and Datta et al (1990) Plant Cell Rep. 9:253, and protoplasts (when obtainable) can generally be obtained from the embryogenic suspension cultures, for example, according to the procedures described by Li and Murai (1990) Plant Cell Rep. 9:216. However under conventional culture conditions as described, for example, in Shimamoto et al (1989) supra, Datta et al (1990) supra and Hayashimoto et al (1990) supra, it is practically impossible to regenerate phenotypically normal (e.g., fertile) plants from embryogenic suspension cultures or protoplasts of such rice lines.

This invention also provides novel transgenic corn plants of corn lines, from which embryogenic suspension cultures (when obtainable) can generally be obtained, for example, according to the procedures described by Shillito et al (1989) Bio/Technology 7:581, Prioli and Söndahl (1989) Bio/Technology 7:589, Gordon-Kamm et al (1990) supra, and Fromm et al (1990) supra, and protoplasts (when obtainable) can generally be obtained from such embryogenic suspension cultures, for example, according to the procedures described by Shillito et al (1989) supra and Prioli and Söndahl (1989) supra. However under conventional culture conditions as described, for example, by Shillito et al (1989) supra, Prioli and Söndahl (1989) supra, Gordon-Kamm et al (1990) supra and Fromm et al (1990) supra, it is practically impossible to regenerate phenotypically normal (e.g., fertile) plants from embryogenic suspension cultures or protoplasts of such corn lines. Furthermore, such corn lines have the capacity to form type I callus at high frequencies but do not possess the ability to form type II callus at frequences higher than 10%, particularly at frequencies higher than 1%, quite particularly at frequencies higher than 0.1%, more quite particularly at frequencies higher than

0.01. Type II corn callus is the only type of corn
callus tissue, from which embryogenic suspension
cultures and regenerable protoplasts can be suitably
obtained that can be stably transformed, and thus, the
inability to obtain type II callus for a particular
corn line has meant, up to now, that one could not
regenerate phenotypically normal (e.g., mature),
transgenic corn plants from transformed callus of such
corn line. The practical ability to obtain type II
callus from a particular corn line can be assessed by
the general procedures described by Gordon-Kamm et al
(1990) supra and Fromm et al (1990) supra and the
references mentioned therein. In making this
assessment: callus cultures can be initiated from, for
example, 1000 immature embryos of a corn line; the
cultures can be maintained by subculturing every 3
weeks, and only those of the cultures that most
resemble typical type II callus can be subcultured; and
after 6 months, it can be determined at what
frequencies a uniform type II callus is obtained.

More generally, to determine whether it is
practical to obtain regenerable protoplasts from a
specific line of a monocot species, the following well
known procedures can be followed. In this regard, it is
believed that regenerable protoplasts are most
efficiently and reliably generated from embryogenic
suspension cultures which, for any specific monocot,
can be produced and maintained by conventional means.
The extent and quality of an embryogenic suspension
culture is generally dependent on its genotype, and it
is generally only worthwhile to form protoplasts of a
plant line, for transformation, if its embryogenic
suspension culture is capable of plant regeneration.
Embryogenic suspension cultures can generally be
characterized as consisting of well dispersed, small

groups of richly cytoplasmic embryogenic cells, as
being free of callus tissues or organized meristems, as
having cell doubling times of 27-32 hours, and as being
capable of forming somatic embryos and plants (Vasil
(1988) Bio/Technology 6:397). It can be determined
whether an embryogenic suspension culture of a
particular line of a monocot species is suitable for
plant regeneration by plating a large number (i.e., at
least 100) of cell aggregates on a suitable
regeneration medium and determining what proportion of
the aggregates give rise to phenotypically normal,
fertile plants. If normal fertile plants are obtained
from 50% or more of the cell aggregates, it is
generally considered worthwhile to proceed with
protoplast generation. However, a specific monocot line
can be considered as not being suitable for providing
regenerable protoplasts suitable for plant
transformation if, using conventional protoplast
isolation, culture, and plant regeneration techniques:
for every 10,000 protoplasts, no more than about 500,
especially no more than about 100, particularly no
more than about 10, quite particularly no more than
about 1, phenotypically normal (e.g., fertile) plant(s)
can be regenerated.

The Examples, which follow, illustrate this
invention. Unless otherwise indicated, all experimental
procedures for manipulating recombinant DNA were
carried out by the standardized procedures described in
Sambrook et al (1990) Molecular Cloning: A Laboratory
Manual, Cold Spring Harbor Laboratory. Oligonucleotides
were designed according to the general rules outlined
by Kramer and Fritz (1968) Methods in Enzymology
154:350 and synthesized by the phosphoramidite method
of Beaucage and Caruthers (1981) Tetrahedron Letters
22:1859 on an Applied Biosystems 380A DNA synthesizer

(Applied Biosystems B.V., Maarssen, Netherlands). The
following bacterial strains and plasmids, used in the
Examples, are available from the Deutsche Sammlung für
Mikroorganismen und Zellkulturen ("DSM"), Mascheroder
Weg 1B, Braunschweig, Germany:

| Bacterial strain | plasmid | DSM No | Date of Deposit |
|---|---|---|---|
| E. coli WK6 | pMa5-8 | DSM 4567 | May 3, 1988 |
| E. coli WK6 | pMc5-8 | DSM 4566 | May 3, 1988 |

**Example 1: Transformation of corn with a selectable
marker gene by electroporation of DNA into zygotic
immature embryos**

Zygotic immature embryos of about 0.5 to 1 mm were
isolated from developing seeds of two corn inbred
lines, Pa91 and H99. The freshly isolated embryos were
enzymatically treated for 1-2 minutes with an enzyme
solution II (0.3% macerozyme (Kinki Yakult,
Nishinomiya, Japan) in CPW salts (Powell & Chapman
(1985) "Plant Cell Culture, A Practical Approach", R.A.
Dixon ed., Chapter 3) with 10% mannitol and 5 mM 2-[N-
Morpholino] ethane sulfonic acid (MES), pH 5.6). After
1-2 minutes incubation in this enzyme solution, the
embryos were carefully washed with N6aph solution
(macro- and micro-elements of N6 medium (Chu et al
(1975) Sci. Sin. Peking 18:659) supplemented with 6mM
asparagine, 12 mM proline, 1 mg/l thiamine-HCl, 0.5
mg/l nicotinic acid, 100 mg/l casein hydrolysate, 100
mg/l inositol, 30 g/l sucrose and 54 g/l mannitol).
After washing, the embryos were incubated in the maize
electroporation buffer, EPM-NaCl (150 mM NaCl, 5 mM
CaCl$_2$, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-
ethanesulfonic acid) and 0.425 M mannitol, pH 7.2).

Approximately 100 embryos in 200 μl EPM-NaCl were
loaded in each cuvette. About 20 μg of a plasmid DNA,
pDE108 linearized with HindIII, were added per cuvette.
pDE108 is a 5399 bp plasmid, the entire sequence of
which is set forth in Seq. Id. No. 1 and which contains
a chimaeric gene comprising the kanamycin resistance
gene (neo) under the control of the 35S3 promoter (EP
359617).

After 1 hour DNA incubation with the explants, the
cuvettes were transferred to an ice bath. After 10
minutes incubation on ice, the electroporation was
carried out: one pulse with a field strength of 375
V/cm was discharged from a 900 μF capacitor. The
electroporation apparatus was as described by Dekeyser
et al (1990) The Plant Cell 2:591. Immediately after
electroporation, fresh liquid N6aph substrate was added
to the explants in the cuvette, after which the
explants were incubated for a further 10 minute period
on ice.

Afterwards, the embryos were transferred to Mahl
VII substrate (macro- and micro-elements and vitamins
of N6 medium supplemented with 100 mg/l casein
hydrolysate, 6 mM proline, 0.5 g/l MES, 1 mg/l 2,4-
dichlorophenoxyacetic acid (2,4-D) and 2% sucrose
solidified with 0.75 g/l MgCl$_2$ and 1.6 g/l Phytagel
(Sigma Chemical Company, St Louis, Mo. U.S.A.), pH 5.8)
and supplemented with 0.2M mannitol. After 3 days for
line H99 and 2 days for line Pa91, the embryos were
transferred to the same substrate supplemented with 200
mg/l kanamycin. After approximately 14 days, the
embryos were transferred to Mahl VII substrate without
mannitol, supplemented with kanamycin. The embryos were
further subcultured on this selective substrate for
approximately 2 months with subculturing intervals of
about 3 weeks. The induced embryogenic tissue was

carefully isolated and transferred to MS medium
(Murashige and Skoog (1962) Physiol. Plant 15:473)
supplemented with 5 mg/l 6-benzylaminopurine for line
H99 and 5 mg/l zeatin for line Pa91. The embryogenic
tissue was maintained on this medium for approximately
14 days and subsequently transferred to MS medium
without hormones and 6% sucrose for line H99 and 3%
sucrose for line Pa91. Developing shoots were
transferred to 1/2 MS medium with 1.5% sucrose for
further development to normal plantlets. These
plantlets were transferred to soil and cultivated in
the greenhouse.

## Example 2: Characterization of the transformed corn plants of Example 1

Seventeen plants from Example 1 were analysed for
the presence of the chimaeric neo gene by means of the
polymerase chain reaction (PCR). DNA was prepared
according to the protocol described by Dellaporta et al
(1983) Plant Mol. Biol. Reporter 1:19, adapted for
application to tissue amounts of about 10 to 20 mg. For
each plant, such an amount of tissue was macerated in
extraction buffer in a microfuge tube. A 706 bp
fragment, corresponding to part of the coding sequence
of the neo gene, was amplified with the polymerase
chain reaction according to the protocol described by
Sambrook et al (1990) supra, using oligonucleotide
probes complementary to the sequences of plasmid pDE108
from nucleotide 1384 to 1406 and 2089 to 2067
(numbering as in Seq. Id. No. 1). In total, 35 cycles
with an annealing temperature of 50°C were carried out.
The final DNA was analysed by electrophoresis on a 1.5%
agarose gel. A 706 bp fragment could be identified in a
total of 13 plants. One of the positive plants died at
a later stage.

Activity of the expression product of the neo gene
(i.e., neomycin phosphotransferase II (NPTII)) was
assayed in 9 of the plants as follows. Crude extracts
were prepared by grinding plant tissue in extraction
buffer (McDonnell et al (1987) Plant Molecular Biol.
Reporter 5:380). The extracts were then subjected to
non-denaturing polyacrylamide gel electrophoresis
according to the procedure described by Reiss et al
(1984) Gene 30:211. NPTII activity was then assayed by
in situ phosphorylation of kanamycin using
[gamma-$^{32}$P]ATP as a substrate (McDonnell et al (1987)
supra). NPTII activity was found in 8 of the plants
that were examined (Figure 1).

One of the plants (H99-M148-1), that was found to
be positive on both the PCR and NPTII assay, was
further analyzed by means of Southern hybridization.
Genomic DNA was prepared from plant tissue according to
the protocol described by Dellaporta et al (1983) supra
supplemented by a treatment with RNase to remove
remaining RNA. A non-transformed H99 plant was used as
a control. Samples of the DNA were digested with one of
the following restriction enzymes: BglII, EcoRI, EcoRV,
HindIII, BamHI, PvuI, PvuII or PstI and subjected to
horizontal agarose electrophoresis. Southern transfer
to Hybond N+ (Amersham International PLC, Amersham,
United Kingdom) membranes by means of the "alkali
blotting of DNA" protocol and the subsequent
hybridization were performed as recommended by the
manufacturer (Amersham Hybond-N+ leaflet). Radioactive
probes were prepared with the multi-prime DNA labelling
kit (Amersham) according to the protocol supplied by
the manufacturer which was derived from published
procedures (Feinberg and Vogelstein (1983) Anal.
Biochem. 132:6). As a probe, a 1184 bp EcoRI-HindIII
fragment derived from another plasmid was used. The

sequence of this plasmid is given in Seq. Id. No. 2. The banding patterns (e.g., see Figure 2) showed that at least the chimaeric neo gene was integrated into the plant genomic DNA.

Further analysis of this transformed plant (H99-m148-1) showed that it carries two almost intact copies of the plasmid pDE108 and part of a third rearranged copy. The two almost complete copies are apparently inserted in the plant genome in a head to tail concatamer configuration. However, some rearrangements must have occurred as an additional NcoI site and an additional BglII site were created, while the HindIII site (used for linearization of pDE108 prior to electroporation) at the junction of the two copies was lost. Sequencing of the junction of the two plasmid copies, as integrated in the plant genome, revealed that only the protruding 5' termini of the HindIII site are missing, thus creating a NcoI site as follows:

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AGCCA        +      AGCTTGGCG      =      AGCCATGGCG
TCGGTTCGA           ACCGC                 TCGGTACCGC
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(the lost bases are underlined, and the created NcoI site at the junction is highlighted). Additional analysis showed that no or very few plasmid DNA sequences around the HindIII sites, flanking the plant genome, were lost. Although the other plants were not tested in this way, the PCR and NPTII assays showed that the chimaeric genes are present and expressed.

The mature transformed plants were fertile and phenotypically completely normal. The plant that was previously assayed by Southern hybridization was used as pollinator plant in three crossings with untransformed plants (two from corn inbred line H99 and one from corn inbred line Pa91). A total of 44 of the

plants of the F1 progeny were assayed for NPTII activity as described above, and twenty of them were found to be positive. This does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation assuming that the transformed pollinator plant had one active copy (or alternatively, multiple closely linked active copies) of the chimaeric neo gene ($X^2$ = 0.36).

**Example 3 : Transformation of corn with a selectable marker gene by electroporation of DNA into type I callus derived from immature zygotic embryos**

Immature zygotic embryos of about 0.5 to 1 mm in length were isolated from developing seeds of the corn inbred line Pa91 and cultured on Mahl VII substrate with subsequent subculture intervals of about 14 days. Embryogenic tissue was carefully dissected out from developing type I callus. The embryogenic tissue in EPM (EPM-NaCl without NaCl) was then finely cut in fragments with a maximum length of about 1.5 mm. The resulting callus fragments were preplasmolysed for three hours in this buffer. After three hours, the callus fragments were transferred to EPM-NaCl. About 100-150 mg of callus fragments were transferred to 200 µl EPM-NaCl per cuvette. 20 µg DNA of plasmid pDE108 (Seq. Id. No. 1), linearized with HindIII, was added to each cuvette. The DNA was incubated with the callus fragments for one hour, after which the cuvettes were transferred to an ice bath.

After 10 minutes incubation on ice, the electroporation was carried out: one pulse with a field strength of 375 V/cm was discharged from a 900 µF capacitor. The electroporation apparatus was as described by Dekeyser et al (1990) supra. Immediately after electroporation, fresh liquid N6aph substrate,

supplemented with 6mM asparagine, 12 mM proline, 1 mg/l
thiamine-HCl, 0.5 mg/l nicotinic acid, 100 mg/l casein
hydrolysate, 100 mg/l inositol, 30 g/l sucrose and 54
g/l mannitol, was added to the callus fragments which
were then further incubated for 10 minutes on ice.

After one day culture in liquid N6aph substrate
supplemented with 1 mg/l 2,4-D, the callus fragments
were transferred to Mahl VII substrate supplemented
with 0.2M mannitol and 200 mg/l kanamycin. Fourteen
days later, the callus fragments were subcultured on
the same selective substrate but without mannitol and
further cultured on this substrate for about 2 months
with subculturing intervals of about 3 weeks. The
embryogenic sectors of the resulting calli were
isolated from the slimy tissue and transferred to MS
substrate (Murashige and Skoog (1962) Physiol. Plant
15:473) with 3% sucrose and supplemented with 5 mg/l
zeatin to germinate. Tissue was maintained on this
medium for approximately 2 weeks and subsequently
transferred to MS medium with 3% or 6% sucrose. Shoots
that developed on this substrate were transferred to
half-strength MS medium with 1.5% sucrose for further
development to normal plantlets. These plantlets were
transferred to soil and cultivated in the greenhouse.

**Example 4: Characterization of the transformed corn
plants of Example 3**

Twenty nine plants from Example 3 were analysed
for the presence of the chimaeric neo gene by means of
the polymerase chain reaction. DNA was prepared
according to Dellaporta et al (1983) Plant Mol. Biol.
Reporter 1:19, adapted for application to tissue
amounts of about 10 to 20 mg. For each plant, such an
amount of tissue was macerated in extraction buffer in
a microfuge tube. A 706 bp fragment, corresponding to

part of the coding sequence of the neo gene, was amplified with the polymerase chain reaction according to the protocol described by Sambrook et al (1990) supra, using oligonucleotide probes complementary to the sequences of plasmid pDE108 from nucleotide 1384 to 1406 and 2089 to 2067 (numbering as in Seq. Id. 1). In total, 35 cycles with an annealing temperature of 50C were carried out. The final DNA was analysed by electrophoresis on a 1.5% agarose gel. A 706 bp fragment could be identified in a total of 14 plants. One of the positive plants died at a later stage.

Activity of the NPTII expression product of the neo gene was assayed in 24 of the plants as follows. Crude extracts were prepared by grinding plant tissue in extraction buffer (McDonnell et al (1987) Plant Molecular Biol. Reporter 5:380). The extracts were then subjected to non-denaturing polyacrylamide gel electrophoresis according to the procedure described by Reiss et al (1984) Gene 30:211. NPTII activity was then assayed by in situ phosphorylation of kanamycin using [gamma-32P]ATP as a substrate (McDonnell et al, supra). NPTII activity was found in 14 of the plants that were examined (Figure 3). Two plants that were NPTII positive scored negative in a PCR assay.

Two of the plants (Pa91-M146-2 and Pa91-M149-1), that were found to be positive on both the PCR and NPTII assays, were further analyzed by means of Southern hybridization. Genomic DNA was prepared from plant tissue according to Dellaporta et al (1983) supra, supplemented by a treatment with RNase to remove remaining RNA. A non-transformed Pa91 plant was used as control. Samples of the DNA were digested with one of the following restriction enzymes: BglII, EcoRI, EcoRV, HindIII, BamHI, PvuI, PvuII or PstI and subjected to horizontal agarose electrophoresis. Southern transfer

to Hybond N+ membranes by means of the "alkali blotting of DNA" protocol and the subsequent hybridization were performed as recommended by the manufacturer (Amersham). Radioactive probes were prepared with the multi-prime DNA labelling kit (Amersham) according to the protocol supplied by the manufacturer which was derived from published procedures (Feinberg and Vogelstein (1983) Anal. Biochem. 132:6). As a probe, a 1184 bp EcoRI-HindIII fragment derived from another plasmid was used. The sequence of this plasmid is given in Seq. Id. No. 2. The banding patterns (e.g., see Figure 4) showed that at least the chimaeric neo gene was integrated into the plant genomic DNA.

Further analysis of one of the transformed plants (Pa91-M146-2) showed that it carried two almost intact copies of the plasmid pDE108 in a head to tail configuration. The HindIII site (used for linearization of pDE108 prior to electroporation) at the junction of the two copies was lost. Sequencing of the junction of the two plasmid copies, as integrated in the plant genome, revealed that the protruding 5' termini of the HindIII site plus one base downstream of one of the HindIII sites are missing as follows:

AGCCA      +      AGCTTGGCG      =      AGCCAGGCG
TCGGTTCGA             ACCGC             TCGGTCCGC

(the lost bases are underlined). Additional analysis showed that no or very few plasmid DNA sequences around the HindIII sites, flanking the plant genome, were lost. Although the other plants were not tested in this way, the PCR and NPTII assays showed that the chimearic genes are present and expressed.

The adult plants were fertile and phenotypically completely normal. One of the plants, previously

assayed by Southern hybridization, was used as a pollinator plant in a crossing with an untransformed plant from the corn inbred line H99. A total of 20 plants of the F1 progeny were assayed for NPTII activity as described above, and six of them were found to be positive. This does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation assuming that the transformed pollinator plant had one active copy of the chimaeric neo gene ($X^2$ = 3.2).

## Example 5: Transformation of corn with a male-sterility gene and a selectable marker by gene electroporation of DNA into zygotic immature embryos

Zygotic immature embryos of about 1 to 1.5 mm were isolated from developing seeds of corn inbred line H99. Freshly isolated embryos were enzymatically treated and washed as described in Example 1. After washing, the embryos were loaded in the maize electroporation buffer, EPM-KCl (80 mM KCl, 5 mM $CaCl_2$, 10 mM HEPES and 0.425 M mannitol, pH 7.2). Approximately 100 embryos in 200 µl EPM-KCl were loaded in each cuvette. About 20 µg of a plasmid DNA, pVE107 linearized with HindIII, were added per cuvette. pVE107 is a 6659 bp plasmid which is obtained by ligation of the 1287 bp EcoRV-EcoRI fragment of pTTM8 (EP 344029; Seq. Id. No. 3) to the large XbaI (filled-in with Klenow)-EcoRI fragment of plasmid pDE108 (Seq. Id. No. 1). pVE107 contains: a chimaeric gene comprising the kanamycin resistance gene (neo) under the control of the 35S3 promoter; and another chimaeric gene comprising the barnase gene (Hartley (1988) J. Mol. Biol. 202:913) under the control of the tapetum-specific promoter of the TA29 gene of Nicotiana tabacum (EP 344029).

All vector constructions involving fragments of
the barnase gene were carried out in E. coli strain WK6
containing the plasmid pMc5BS. pMc5BS contains the
barstar gene (encoding an inhibitor of barnase) under
the control of the tac promoter (De Boer et al (1983)
Proc. Natl. Acad. Sci. USA 80:21). This plasmid is
constructed by: cloning the EcoRI-HindIII fragment of
plasmid pMT416 (see Hartley (1988) supra) into the
EcoRI and HindIII sites of plasmid pMc5-8 (DSM 4566);
and then deleting the sequence, starting with the
initiation codon of the phoA signal sequence and ending
with the last nucleotide before the translation
initiation codon of the barstar coding region, by means
of a looping-out mutagenesis procedure as generally
described by Sollazo et al (1985) Gene 37:199.

After a 1 hour DNA incubation with the explants,
the cuvettes were transferred to an ice bath. After 10
minutes incubation on ice, the electroporation was
carried out as described in Example 1. Immediately
after electroporation, fresh liquid N6aph substrate was
added to the explants in the cuvette, after which the
explants were incubated for a further 10 minute period
on ice.

Afterwards, the embryos were transferred to Mah1
VII substrate supplemented with 0.2 M mannitol and 200
mg/l kanamycin. After approximately 14 days, the
embryos were transferred to Mah1 VII substrate without
mannitol but with the same selective agent, kanamycin.
The embryos were further subcultured on this selective
substrate for approximately 2 months, with subculturing
intervals of about 3 to 4 weeks. The induced
embryogenic tissue was carefully isolated and
transferred to MS medium (Murashige and Skoog (1962)
supra) supplemented with 5 mg/l 6-benzylaminopurine.
The embryogenic tissue was maintained on this medium

for approximately 14 days and subsequently transferred
to MS medium without hormones and sucrose. Developing
shoots were transferred to 1/2 MS medium with 1.5%
sucrose for further development to normal plantlets.
These plantlets were transferred to soil and cultivated
in the greenhouse.

## Example 6: Characterization of the transformed corn plants of Example 5

Seven plants from Example 5, designated RZM19-2,
RZM19-3, RZM19-4, RZM19-5, RZM19-6, RZM19-7 and
RZM19-8, were derived from the same embryogenic callus
clump. They were subjected to extensive Southern
analysis. In this regard, BamHI-NcoI digested genomic
DNA of the plants was probed with pVE107 and with the
small EcoRV-XbaI fragment of pTTM8 (containing PTA29-
barnase; see Seq. Id. No. 3). In all plants, the
strongest detected band was the expected 1400 bp
fragment. However, the pattern found in these and other
southern blots was very complex and indicated that
transformation had resulted in many insertions of all
or part of pVE107 into the plants' genomes. Some of the
inserted copies of pVE107 were apparently incomplete
and/or had undergone rearrangements. However, the same
complex integration pattern was found in all seven
plants. This could be explained by the fact that the
seven transformants were all derived from one
embryogenic callus clump.

The transformed plants were male sterile but
otherwise phenotypically completely normal; female
fertility, for instance, was normal. The spikelets of
the male flowers were of about normal length but were
very thin and appeared to be empty, and they never
opened. A detailed analysis showed that the anthers
were reduced to almost microscopic structures. This

phenotype indicates not only that at least one copy of the barnase gene was expressed but also that it was selectively expressed in some or all of the tissues of the anthers.

Transformant RZM19-3 was pollinated with pollen from an untransformed H99 plant, and 53 progeny plantlets were recovered. Of these 53 plantlets, 32 (60%) scored positive in a NPTII assay, while 21 (40%) were NPTII negative. This proportion in the F1 progeny does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation assuming that the transformed female parent had one active copy of the chimaeric neo gene ($X^2$ = 2.28). The NPTII negative progeny were male fertile, while the NPTII positive progeny were male sterile.

31 NPTII positive progeny plants were subjected to Southern analysis. 28 of these plants displayed the same integration pattern as that of the original transformant, RZM19-3, from which they were derived. 3 plants had a slightly altered pattern.

## Example 7: Transformation of corn with a male-sterility gene and a herbicide resistance gene by electroporation of DNA into zygotic immature embryos

Zygotic embryos of corn inbred line H99 were isolated, enzymatically treated, washed, and loaded in electroporation buffer as described in Example 5. Approximately 100 embryos in 200 μl EPM-KCl were loaded in each cuvette. About 20 μg of a plasmid DNA, pVE108 linearized with HindIII, was added per cuvette. pVE108 is a 5620 bp plasmid which is obtained by ligation of the 1287 bp EcoRV-EcoRI fragment of pTTM8 (EP 344029; Seq. Id. No. 3) to the large EcoRI-StuI fragment of plasmid pDE110 (Seq Id. No. 4). pVE108 contains: a chimaeric gene comprising the bar gene (EP 242236),

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encoding phosphinothricin acetyl transferase (PAT) and conferring resistance to an herbicidal glutamine synthetase inhibitor such as phosphinothricin (PPT), under the control of the 35S3 promoter; and another chimaeric gene comprising the barnase gene (Hartley (1988) supra) under the control of the tapetum-specific promoter of the TA29 gene (EP 344029) of N. tabacum. All vector constructions involving DNA fragments comprising the barnase gene were carried out in E. coli strain WK6 containing the plasmid pMc5BS of Example 5.

After a 1 hour DNA incubation with the explants, the cuvettes were transferred to an ice bath. After 10 minutes incubation on ice, the electroporation was carried out as described in Example 1. Immediately after electroporation, fresh liquid N6aph substrate was added to the explants in the cuvette, after which the explants were incubated for a further 10 minute period on ice.

Afterwards, the embryos from one electroporation experiment were transferred to Mahl VII substrate supplemented with 0.2 M mannitol and 2 mg/l PPT. After approximately 14 days, the embryos were transferred to Mhl VII substrate (Mahl VII substrate of Example 1 but without proline and casein hydrolysate) supplemented with 2 mg/l PPT but without mannitol. After approximately 4 weeks, the embryos were subcultured for another month on Mhl VII substrate supplemented with 10 mg/l PPT. The induced embryogenic tissue was carefully isolated and transferred to MS medium supplemented with 5 mg/l 6-benzylaminopurine. The embryogenic tissue was maintained on this medium for approximately 14 days and subsequently transferred to MS medium without hormones and sucrose. Developing shoots were transferred to 1/2 MS medium with 1.5% sucrose for further development to normal plantlets. These plantlets survived an in vitro

spraying with doses of BASTA[R] (Hoechst AG, Frankfurt am
Main, Germany) corresponding to 2 l/ha. These plantlets
were then transferred to soil and cultivated in the
greenhouse, and two of the transformed plantlets,
designated RZM35-1 and RZM35-18, were further
characterized (see Example 8).

The embryos from a second eletroporation
experiment were transferred to Mhl VII substrate
supplemented with 2 mg/l PPT and 0.2 M mannitol. After
about 14 days, the embryos were transferred to Mhl VII
substrate supplemented with 2 mg/l PPT but without
mannitol. After approximately another three weeks, the
embryos were transferred to Mhl VII substrate
supplemented with 10 mg/l PPT but without mannitol.
After another three weeks, the induced embryogenic
tissue was carefully isolated and transferred to MS
medium supplemented with 2 mg/l PPT and 5 mg/l 6-
benzylaminopurine. The embryogenic tissue was
maintained on this medium for approximately 14 days and
subsequently transferred to MS medium without hormones,
sucrose or PPT. Developing shoots were transferred to
1/2 MS medium with 1.5% sucrose for further development
to normal plantlets. The resulting plantlets were
transferred to soil and cultivated in the greenhouse,
and three of the transformed plantlets, designated
RZM34-1, RZM34-12, and RZM34-14, were further
characterized (see Example 8).

Example 8: Characterization of the transformed corn
plants of Example 7

RZM34-1, RZM34-12, RZM34-14, RZM35-1, and RZM35-18
of Example 7 were grown in the greenhouse. Activity of
the expression product of the bar gene in leaves of the
plants was assayed as follows in a "PAT assay". 100 mg
of leaf tissue from each plant, together with 50 mg of

acid-treated sea sand (Merck, Darmstadt, Germany) and 5
mg polyvinylpolypyrrolidone (PVPP), were ground in an
Eppendorf tube with a glass rod in 50 $\mu$l of extraction
buffer (25 mM Tris-HCL pH 7.5, 1 mM Na$_2$-EDTA
(ethylenediaminetetraacetic acid disodium salt), 0.15
mg/ml phenylmethylsulfonylfluoride (PMSF), 0.3 mg/ml
dithiothreitol (DTT), and 0.3 mg/ml bovine serum
albumin). The extract was centrifuged in a microfuge
for 5 minutes at 16000 rpm. The supernatant was
recovered and diluted ten times with TE 25/1 (25 mM
Tris-HCL pH 7.5, 1 mM Na$_2$-EDTA. To twelve $\mu$l of the
diluted extract was then added: 1$\mu$l of 1 mM PPT in TE
25/1, 1 $\mu$l of 2 mM ActeylCoenzyme A in TE 25/1, and 2
$\mu$l of [$^{14}$C]AcetylCoenzym A (60 mCi/mmol, 0.02 mCi/ml,
[NEN Research Products, DUPONT, Wilmington, Delaware,
USA). The reaction mixture was incubated for 30 minutes
at 37°C and spotted on a aluminium sheet silicagel 60
t.l.c. plate with concentrating zone (Merck). Ascending
chromatography was carried out in a 3 to 2 mixture of
1-propanol and NH$_4$OH (25% NH$_3$). $^{14}$C was visualized by
overnight autoradiography (XAR-5 Kodak film).

The tolerance to the herbicide BASTA$^R$ was tested
by brushing a small area near the top of one leaf per
plant with a 1% solution of the herbicide and observing
the damage symptoms at and near the brushed sites.
While RZM34-1, RZM35-1 and RZM35-18 showed no damage
symptoms at all, RZM34-12 and RZM34-14 displayed slight
browning and drying-out of the brushed site.

RZM34-1, RZM34-12, RZM34-14, RZM35-1 and RZM35-18
were also shown to be male sterile. The phenotype of
each of these plants was identical to that described
for the transformants of Example 5 which were analyzed
in Example 6.

Southern analysis showed RZM35-1 and RZM35-18 to
have an identical integration pattern, with only one

copy of plasmid pVE108 being present in the genome of each. A small part of the plasmid DNA sequence adjacent to the HindIII site (used for linearization prior to electroporation) seemed to be absent in the integrated copy. Southern analysis of RZM34-1, RZM34-12 and RZM34-14 showed that each of these plants probably has two or three copies of part or all of pVE108 integrated into its genome. The copies are most likely not inserted in a concatemer configuration.

Transformants RZM35-1 and RZM34-1 were pollinated with pollen from an untransformed H99 plant and progeny plantlets were recovered. From the 35 plantlets recovered from RZM35-1, 16 (46%) scored positive in a PAT assay, while 19 (54%) were PAT negative. This proportion in the F1 progeny does not differ significantly from the 1:1 ratio expected under normal Mendelian segregation of one active copy of the chimaeric bar gene ($X^2 = 0.26$).

From the 34 plantlets recovered from RZM34-1, 19 (56%) scored positive in a PAT assay, while 15 (44%) were PAT negative. This proportion in the F1 progeny does not differ significantly from the 1:1 ration expected under normal Mendelian segregation assuming that the transformed female parent had one active copy, or alternatively multiple active, but closely linked copies, of the chimaeric bar gene ($X^2 = 0.47$).

## Example 9: Transformation of rice with a herbicide resistance gene by electroporation of DNA into compact embryogenic callus derived from dry seeds

Dehusked mature seeds of the rice cultivar Nipponbare were surfaced-sterilized, placed on solid 2N6 medium (N6 medium (Chu et al (1975) supra), supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 1.0 mg/l thiamine-HCl, 2.0 mg/l 2, 4-D,

30 g/l sucrose, and 2.0 g/l Phytagel, pH 5.8), and
cultured at 27°C in the dark. Callus developed from the
scutella of the embryos within 3-4 weeks. Embryogenic
portions of primary callus were transferred to N67
medium (N6 medium (Chu et al (1975) supra),
supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l
pyridoxine-HCl, 1.0 mg/l thiamine-HCl, 2.0 g/l casamino
acids (vitamin assay, Difco), 1.0 mg/l 2,4-D, 0.5 mg/l
6-benzylaminopurine, 20 g/l sucrose, 30g/l sorbitol,
and 2.0 g/l Phytagel, pH 5.8) for propagation into
compact embryogenic callus.

Three to four weeks after subculture, the
embryogenic callus was used for transformation
experiments. The callus was cut into fragments with a
maximum length of about 1.5 to 2 mm. The callus pieces
were washed twice in EPM and then preplasmolyzed in
this buffer for 30 minutes to 3 hours at room
temperature (25°C). Then, the callus fragments were
washed twice with EPM-KCl and transferred to
electroporation cuvettes. Each cuvette was loaded with
about 150 to 200 mg of callus fragments in 100 to 200
μl EPM-KCl. 10 to 20 μg of a plasmid DNA, either
circular pDE110 or pDE110 linearized with HindIII or
EcoRI, were added per cuvette. pDE110 is a 4883 bp
plasmid, the entire sequence of which is set forth in
Seq. Id. No. 4 and which contains a chimaeric gene
comprising the bar gene under the control of the 35S3
promoter.

The DNA was incubated with the callus fragments
for about 1 hour at room temperature. Electroporation
was then carried out as described in Example 1. After
electroporation, liquid N67 medium without casamino
acids was added to the callus fragments. The callus
fragments were then plated on solid N67 medium without
casamino acids but supplemented with 5, 10 or 20 mg/l

PPT and were cultured on this selective medium at 27°C under a light/dark regime of 16/8 hours for about 4 weeks. Developing PPT-resistant calli were isolated and subcultured for about two to three weeks onto fresh N67 medium without casamino acids but containing 5 mg/l PPT. Thereafter, selected PPT-resistant calli were transferred to plant regeneration medium N6M25 (N6 medium (Chu et al (1975) supra), supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine-HCl, 1.0 mg/l thiamine-HCl, 288 mg/l aspartic acid, 174 mg/l arginine, 7.0 mg/l glycine, 1.0 mg/l O-naphthalenacetic acid (NAA), 5.0 mg/l kinetin, 20 g/l sucrose and 2.0 g/l Phytagel, pH 5.8) supplemented with 5 mg/l PPT. Plantlets developed within approximately 1 month and were then transferred to hormone-free N6 medium (Chu et al (1975) supra), supplemented with 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxin-HCl, 1.0 mg/l thiamine-HCl, 1.0 g/l casamino acids, 20 g/l sucrose, and 2.0 g/l Phytogel, pH 5.8) on which they were kept for another 2 to 3 weeks, after which they were transferred to soil and cultivated in the greenhouse.

The compositions of the 2N6, N67, N6M25 and hormone-free N6 media, described above, were kindly provided by Japan Tobacco Inc., Plant Breeding and Genetics Research Laboratory, 700 Higashibara, Toyoda, Iwata, Shizuoka 438, Japan.

## Example 10: Characterization of the transformed rice plants of Example 9

Two transformed rice plants of Example 9, obtained in different transformation experiments, were cultivated for four weeks in soil and were then sprayed with BASTA[R] at a dosage corresponding to 2 l/ha. The two plants were BASTA[R] resistant and survived the herbicide treatment whereas non-transformed control

plants turned brown and died within four days of herbicide spraying.

The two plants and four other _in vitro_ plantlets, derived from two further transformation experiments of Example 9, were analyzed by means of a Southern hybridization in which plnat genomic DNA, digested with PvuII, was probed with pDE110. This analysis showed that, in all analyzed plants, at least part of one copy of pDE110 was integrated in the rice genome. In five out of six plants, the 1.6 kb fragment coresponding to the pDE110 fragment containing most of the 35S-bar chimaeric gene could be unambiguously identified.

## Example 11: Field tests with the transformed corn plants of Examples 2 and 4

The progeny of the corn transformant H99-M148-1 of Example 2 and of the corn transformant Pa91-M146-2 of Example 4 were tested under field conditions at the Plant Genetic Systems, N.V. experimental farm in Afsnee, Belgium. The field trial was authorized by the Belgian Ministry of Agriculture under registration number BIOT/91/M06. F1, F2, and F3 progeny were obtained from crosses as summarized in Table 1, below. In all cases one of the parents was assumed to be a heterozygote for the neo gene.

Up to 100 seeds of each seedlot were planted in 5 parellel rows, each with a length of 5 meters. Individual plants were 0.25 m apart, and the distance between rows was 1 m. 10 rows of experimental plants were alternated with 1 row of non-transformed H99 and 1 row of non-transformed Pa91 plants as controls. One plot consisted of F1 and F2 experimental plants with controls. Each of these plots was surrounded by i) a path with a width of 1 m and ii) 3 rows (1 m apart) of non-transformed corn plants (variety Sanora).

Experimental plots were prepared, sowed and maintained according to the schedule in Table 2, below. For sowing, plant holes were made with a plant stick, and seeds were put in by hand to a depth of 4 to 5 cm.

The field trial was terminated by manual removal and subsequent steaming of all cobs of the experimental plants. The remainders of the plants were mechanically chopped with a mowing machine.

The following observations were made. At the 2-3 leaf stage, the total number of germinated seeds was counted for each seedlot. As can be seen from Table 3, below, the percentage of germination varied between 63% and 100% with the exception of seedlot P4482, from which only 42% of the seeds germinated. Germination of seedlots of untransformed H99 and Pa91 plants varied between 25% and 75%.

At the 3-4 leaf stage, the phenotype of the transgenic <u>neo</u> gene was assayed as follows. In each plant, an incision up to the midvein was made in two leaves with a small pair of scissors. The incisions were then brushed with a piece of cotton wool drenched in an aqueous suspension of 4% kanamycin and 0.2% SDS. Some plants were treated with a suspension of 5% kanamycin and 0.1% SDS. A plant was scored as sensitive and as lacking an active <u>neo</u> gene when the newly formed leaves were yellow. A plant was scored as resistant and as having an active <u>neo</u> gene when the newly formed leaves were normal and showed no bleaching. Discoloration of the newly formed leaves was assessed about 10 days after the brushing. 5-8% of the tested plants had an intermediate phenotype as they could not be unambiguously scored as sensitive or resistant. This was probably due to variations in environmental conditions and/or developmental stages of the tested

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plants and a less than optimal kanamycin (and/or SDS) concentration.

In later analyses, the intermediate phenotypes were pooled with the sensitive plants. The proportions of kanamycin resistant plants versus kanamycin sensitive plants (including intermediate phenotypes) for each crossing or self was determined by a chi-square goodness of fit test (Snedecor and Cochran (1967) 'Statistical Methods', the Iowa State University Press, Ames, Iowa, U.S.A.) under the assumption of a one locus Mendelian segregation of the neo gene. The results are summarized in Table 3, below.

From the data in Table 3, it can be concluded that the introduced neo gene remained stable over three generations regardless of whether the progeny was obtained through selfing, backcrossing, or outcrossing to an unrelated line. The pattern of segregation was consistent with each original transformant having had only one active copy or multiple closely linked active copies of the neo gene and with the neo gene trait having had a normal Mendelian one-locus inheritance.

In all cases, the experimental plants appeared to be morphologically completely normal when compared to untransformed control plants.

Table 1

| | Cross | Seedlot |
|---|---|---|
| F1 | H99 x H99-M148-1 | P3166 |
| | Pa91 x H99-M148-1 | P3169 |
| | H99 x Pa91-M146-2 | P3162 |
| | Selfing of Pa91-M146-2 | P3173 (1) |
| F2 | P3169-024 x H99 | P3651 |
| | Selfing of P3166-002 | P3989 |
| | P3166-012 x H99 | P3983 |
| | P3166-018 x H99 | P3982 |
| | Selfing of P3173-003 | P3996 |
| | P3162-017 x H99 | P4004 |
| | P3162-008 x Pa91 | P4008 |
| F3 | H99 x (P3166-005 x H99)-001 | P4481 |
| | H99 x (P3162-004 x H99)-011 | P4483 |
| | Selfing of(Selfing of P3166-001)-003 | P4482 |
| | Pa91 x (P3169-028 x Pa91)-004 | P4310 |
| | H99 x (P3169-036 x H99)-003 | P4306 |

(1) not tested

Table 2

| Date | Activity | Quantity |
|------|----------|----------|
| March 29, 1991 | lime treatment of soil | 2000 kg/ha |
| May 23, 1991 | $NH_4NO_3$ treatment | 740 kg/ha |
| May 23, 1991 | superphosphate treatment | 833 kg/ha |
| May 23, 1991 | potassium sulphate | 120 kg/ha |
| May 27, 1991 | sowing of F1 and F2 seedlots | - |
| July 4, 1991 | Herbicide treatment:<br>Laddok<br>paraffin oil | 4 l/ha<br>105 l/ha |
| July 8, 1991 | Sowing of F3 seedlots | - |
| July 26, 1991 | Insecticide treatment:<br>Pyrimor<br>Ambush | 0.265 kg/ha<br>0.133 l/ha |
| October 10, 1991 | termination | - |

Table 3

| | Code | Emerg | % | T | R | I | S | ND | X² | Sign. |
|---|---|---|---|---|---|---|---|---|---|---|
| **F1** | P3169 | 16/20 | 5 | 16 | 5 | 6 | 4 | 1 | 1.67 | n.s. |
| | P3166 | 79/100 | 4 | 79 | 36 | 0 | 35 | 8 | 0.01 | n.s. |
| | P3162 | 86/100 | 4 | 84 | 47 | 1 | 31 | 3 | 2.85 | n.s. |
| **F2** | P3651 | 65/100 | 4 | 62 | 26 | 11 | 16 | 9 | 0.02 | n.s. |
| | P3989 | 91/100 | 4 | 83 | 66 | 1 | 10 | 5 | 4.71 | p<0.05 |
| | P3983 | 36/40 | 4 | 34 | 17 | 2 | 14 | 1 | 0.03 | n.s. |
| | P3982 | 51/60 | 4 | 42 | 20 | 4 | 17 | 1 | 0.02 | n.s. |
| | P3996 | 54/60 | 4 | 48 | 32 | 0 | 11 | 5 | 0.01 | n.s. |
| | P4004 | 92/100 | 4 | 86 | 38 | 11 | 31 | 6 | 0.20 | n.s. |
| | P4008 | 20/20 | 4 | 18 | 6 | 9 | 3 | 0 | 2.00 | n.s. |
| **F3** | P4481 | 72/100 | 5 | 66 | 32 | 2 | 30 | 2 | 0 | n.s. |
| | P4483 | 63/100 | 5 | 47 | 22 | 2 | 23 | 0 | 0.19 | n.s. |
| | P4482 | 42/100 | 5 | 34 | 30 | 0 | 4 | 0 | 3.18 | n.s. |
| | P4310 | 84/100 | 5 | 82 | 50 | 7 | 24 | 1 | 4.46 | p<0.05 |
| | P4306 | 85/100 | 5 | 79 | 39 | 1 | 39 | 0 | 0.01 | n.s. |

Code = seedlot (see Table 1); Emerg = number of seedlings per number of sowed seeds; % = percentage of kanamycin in solution used in brushing assay; T = total number of plants tested; R = number of kanamycin resistant plants; I = number of intermediate phenotypes; S = number of kanamycin sensitive plants; ND = number of tested plants that were not scored because seedlings were stopped in growth and died; $X^2$ = value of chi-square for segregation of R versus I+S (expected values in outcrossings are 50% R - 50% I+S; expected values in selfings are 75% R - 25% I+S under assumption of one locus segregation).

SEQUENCE LISTING

1. General Information

i)      APPLICANT : PLANT GENETIC SYSTEM N.V.

ii)     TITLE   OF   INVENTION   :   Process   for   transforming
        monocotyledonous plants

iii)    NUMBER OF SEQUENCES : 4

iv)     CORRESPONDENCE ADDRESS :
            A. ADDRESSEE : Plant Genetic Systems N.V.
            B. STREET : Plateaustraat 22,
            C. POSTAL CODE AND CITY : 9000 Ghent,
            D. COUNTRY : Belgium

v)      COMPUTER READABLE FORM :
            A. MEDIUM TYPE 5.25 inch, double sided, high density
            1.2 Mb floppy disk
            B. COMPUTER : IBM PC/AT
            C. OPERATING SYSTEM : DOS version 3.3
            D. SOFTWARE : WordPerfect

vi)     CURRENT APPLICATION DATA : Not Available

(vii)       PRIOR APPLICATION DATA :

        European patent application 91401888.2 of July 8, 1991
        European patent application 90403332.1 of November 23, 1990

2. Information for SEQ. ID. NO. 1

i)    Sequence characteristics
            A. TYPE: nucleic acid
            B. LENGTH: 5399 bp
            C. STRANDEDNESS: double stranded
            D. TOPOLOGY: circular

ii)   MOLECULAR TYPE: pDE108 : plasmid DNA replicable in E. coli

ix)   Features:
            1 - 451 :         pUC18 derived sequence
            452 - 1284:       "35S3" promoter sequence derived from
                              Cauliflower mosaic virus isolate CabbB-
                              JI
            1285 - 2100:      coding    sequence    of    neomycine
                              phosphotransferase gene
            2101 - 3160:      3' regulatory sequence containing the
                              polyadenylation   site   derived   from
                              Agrobacterium T-DNA octopine synthase
                              gene
            3161-5399:        pUC18 derived sequence

            Other information: plasmid is replicable in E. coli,
            confers ampicillin resistance to the bacterium

xi)   Sequence description

```
TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG        50
GAGACGGTCA CAGCTTGTCT GTAAGCGGAT GCCGGGAGCA GACAAGCCCG       100
TCAGGGCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG       150
CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA       200
CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCGCCATT       250
CAGGCTGCGC AACTGTTGGG AAGGGCGATC GGTGCGGGCC TCTTCGCTAT       300
TACGCCAGCT GGCGAAAGGG GGATGTGCTG CAAGGCGATT AAGTTGGGTA       350
ACGCCAGGGT TTTCCCAGTC ACGACGTTGT AAAACGACGG CCAGTGAATT       400
CGAGCTCGGT ACCCGGGGAT CCTCTAGAGT CGACCTGCAG GCATGCAAGC       450
TCCTACGCAG CAGGTCTCAT CAAGACGATC TACCCGAGTA ACAATCTCCA       500
GGAGATCAAA TACCTTCCCA AGAAGGTTAA AGATGCAGTC AAAAGATTCA       550
GGACTAATTG CATCAAGAAC ACAGAGAAAG ACATATTTCT CAAGATCAGA       600
AGTACTATTC CAGTATGGAC GATTCAAGGC TTGCTTCATA AACCAAGGCA       650
AGTAATAGAG ATTGGAGTCT CTAAAAAGGT AGTTCCTACT GAATCTAAGG       700
CCATGCATGG AGTCTAAGAT TCAAATCGAG GATCTAACAG AACTCGCCGT       750
GAAGACTGGC GAACAGTTCA TACAGAGTCT TTTACGACTC AATGACAAGA       800
AGAAAATCTT CGTCAACATG GTGGAGCACG ACACTCTGGT CTACTCCAAA       850
AATGTCAAAG ATACAGTCTC AGAAGACCAA AGGGCTATTG AGACTTTTCA       900
ACAAAGGATA ATTTCGGGAA ACCTCCTCGG ATTCCATTGC CCAGCTATCT       950
GTCACTTCAT CGAAAGGACA GTAGAAAAGG AAGGTGGCTC CTACAAATGC      1000
CATCATTGCG ATAAAGGAAA GGCTATCATT CAAGATGCCT CTGCCGACAG      1050
TGGTCCCAAA GATGGACCCC CACCCACGAG GAGCATCGTG GAAAAAGAAG      1100
ACGTTCCAAC CACGTCTTCA AAGCAAGTGG ATTGATGTGA CATCTCCACT      1150
GACGTAAGGG ATGACGCACA ATCCCACTAT CCTTCGCAAG ACCCTTCCTC      1200
TATATAAGGA AGTTCATTTC ATTTGGAGAG GACACGCTGA AATCACCAGT      1250
CTCTCTCTAT AAATCTATCT CTCTCTCTAT AACCATGGAT CCGGCCAAGC      1300
TAGCTTGGAT TGAACAAGAT GGATTGCACG CAGGTTCTCC GGCCGCTTGG      1350
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GTGGAGAGGC TATTCGGCTA TGACTGGGCA CAACAGACAA TCGGCTGCTC    1400
TGATGCCGCC GTGTTCCGGC TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG    1450
TCAAGACCGA CCTGTCCGGT GCCCTGAATG AACTGCAGGA CGAGGCAGCG    1500
CGGCTATCGT GGCTGGCCAC GACGGGCGTT CCTTGCGCAG CTGTGCTCGA    1550
CGTTGTCACT GAAGCGGGAA GGGACTGGCT GCTATTGGGC GAAGTGCCGG    1600
GGCAGGATCT CCTGTCATCT CACCTTGCTC CTGCCGAGAA AGTATCCATC    1650
ATGGCTGATG CAATGCGGCG GCTGCATACG CTTGATCCGG CTACCTGCCC    1700
ATTCGACCAC CAAGCGAAAC ATCGCATCGA GCGAGCACGT ACTCGGATGG    1750
AAGCCGGTCT TGTCGATCAG GATGATCTGG ACGAAGAGCA TCAGGGGCTC    1800
GCGCCAGCCG AACTGTTCGC CAGGCTCAAG GCGCGCATGC CCGACGGCGA    1850
GGATCTCGTC GTGACCCATG GCGATGCCTG CTTGCCGAAT ATCATGGTGG    1900
AAAATGGCCG CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG    1950
GACCGCTATC AGGACATAGC GTTGGCTACC CGTGATATTG CTGAAGAGCT    2000
TGGCGGCGAA TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC    2050
CCGATTCGCA GCGCATCGCC TTCTATCGCC TTCTTGACGA GTTCTTCTGA    2100
GCGGGACTCT GGGGTTCGAA ATGACCGACC AAGCGACGCC CAACCTGCCA    2150
TCACGAGATT TCGATTCCAC CGCCGCCTTC TATGAAAGGT TGGGCTTCGG    2200
AATCGTTTTC CGGGACGCCG GCTGGATGAT CCTCCAGCGC GGGGATCTCA    2250
TGCTGGAGTT CTTCGCCCAC CCCCTGCTTT AATGAGATAT GCGAGACGCC    2300
TATGATCGCA TGATATTTGC TTTCAATTCT GTTGTGCACG TTGTAAAAAA    2350
CCTGAGCATG TGTAGCTCAG ATCCTTACCG CCGGTTTCGG TTCATTCTAA    2400
TGAATATATC ACCCGTTACT ATCGTATTTT TATGAATAAT ATTCTCCGTT    2450
CAATTTACTG ATTGTACCCT ACTACTTATA TGTACAATAT TAAAATGAAA    2500
ACAATATATT GTGCTGAATA GGTTTATAGC GACATCTATG ATAGAGCGCC    2550
ACAATAACAA ACAATTGCGT TTTATTATTA CAAATCCAAT TTTAAAAAAA    2600
GCGGCAGAAC CGGTCAAACC TAAAAGACTG ATTACATAAA TCTTATTCAA    2650
ATTTCAAAAG GCCCCAGGGG CTAGTATCTA CGACACACCG AGCGGCGAAC    2700
TAATAACGTT CACTGAAGGG AACTCCGGTT CCCCGCCGGC GCGCATGGGT    2750
GAGATTCCTT GAAGTTGAGT ATTGGCCGTC CGCTCTACCG AAAGTTACGG    2800
GCACCATTCA ACCCGGTCCA GCACGGCGGC CGGGTAACCG ACTTGCTGCC    2850
CCGAGAATTA TGCAGCATTT TTTTGGTGTA TGTGGGCCCC AAATGAAGTG    2900
CAGGTCAAAC CTTGACAGTG ACGACAAATC GTTGGGCGGG TCCAGGGCGA    2950
ATTTTGCGAC AACATGTCGA GGCTCAGCAG GGGCTCGATC CCCTCGCGAG    3000
TTGGTTCAGC TGCTGCCTGA GGCTGGACGA CCTCGCGGAG TTCTACCGGC    3050
AGTGCAAATC CGTCGGCATC CAGGAAACCA GCAGCGGCTA TCCGCGCATC    3100
CATGCCCCCG AACTGCAGGA GTGGGGAGGC ACGATGGCCG CTTTGGTCGA    3150
CCTGCAGCCA AGCTTGGCGT AATCATGGTC ATAGCTGTTT CCTGTGTGAA    3200
ATTGTTATCC GCTCACAATT CCACACAACA TACGAGCCGG AAGCATAAAG    3250
TGTAAAGCCT GGGGTGCCTA ATGAGTGAGC TAACTCACAT TAATTGCGTT    3300
GCGCTCACTG CCCGCTTTCC AGTCGGGAAA CCTGTCGTGC CAGCTGCATT    3350
AATGAATCGG CCAACGCGCG GGGAGAGGCG GTTTGCGTAT TGGGCGCTCT    3400
TCCGCTTCCT CGCTCACTGA CTCGCTGCGC TCGGTCGTTC GGCTGCGGCG    3450
AGCGGTATCA GCTCACTCAA AGGCGGTAAT ACGGTTATCC ACAGAATCAG    3500
GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA AAAGGCCAGG    3550
AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC TCCGCCCCCC    3600
TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG CGAAACCCGA    3650
CAGGACTATA AAGATACCAG GCGTTTCCCC CTGGAAGCTC CCTCGTGCGC    3700
TCTCCTGTTC CGACCCTGCC GCTTACCGGA TACCTGTCCG CCTTTCTCCC    3750
TTCGGGAAGC GTGGCGCTTT CTCAATGCTC ACGCTGTAGG TATCTCAGTT    3800
CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA ACCCCCCGTT    3850
CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG AGTCCAACCC    3900
GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT AACAGGATTA    3950
GCAGAGCGAG GTATGTAGGC GGTGCTACAG AGTTCTTGAA GTGGTGGCCT    4000
AACTACGGCT ACACTAGAAG GACAGTATTT GGTATCTGCG CTCTGCTGAA    4050
GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC GGCAAACAAA    4100
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CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA GATTACGCGC    4150
AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA CGGGGTCTGA    4200
CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC ATGAGATTAT    4250
CAAAAAGGAT CTTCACCTAG ATCCTTTTAA ATTAAAAATG AAGTTTTAAA    4300
TCAATCTAAA GTATATATGA GTAAACTTGG TCTGACAGTT ACCAATGCTT    4350
AATCAGTGAG GCACCTATCT CAGCGATCTG TCTATTTCGT TCATCCATAG    4400
TTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA GGGCTTACCA    4450
TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT CACCGGCTCC    4500
AGATTTATCA GCAATAAACC AGCCAGCCGG AAGGGCCGAG CGCAGAAGTG    4550
GTCCTGCAAC TTTATCCGCC TCCATCCAGT CTATTAATTG TTGCCGGGAA    4600
GCTAGAGTAA GTAGTTCGCC AGTTAATAGT TTGCGCAACG TTGTTGCCAT    4650
TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG GCTTCATTCA    4700
GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC CATGTTGTGC    4750
AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA GAAGTAAGTT    4800
GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT AATTCTCTTA    4850
CTGTCATGCC ATCCGTAAGA TGCTTTTCTG TGACTGGTGA GTACTCAACC    4900
AAGTCATTCT GAGAATAGTG TATGCGGCGA CCGAGTTGCT CTTGCCCGGC    4950
GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA AAAGTGCTCA    5000
TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT CTTACCGCTG    5050
TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT GATCTTCAGC    5100
ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA GGAAGGCAAA    5150
ATGCCGCAAA AAAGGGAATA AGGGCGACAC GGAAATGTTG AATACTCATA    5200
CTCTTCCTTT TTCAATATTA TTGAAGCATT TATCAGGGTT ATTGTCTCAT    5250
GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA ATAGGGGTTC    5300
CGCGCACATT TCCCCGAAAA GTGCCACCTG ACGTCTAAGA AACCATTATT    5350
ATCATGACAT TAACCTATAA AAATAGGCGT ATCACGAGGC CCTTTCGTC     5399
```

3. Information for SEQ. ID. NO. 2

i)     Sequence characteristics
          A. TYPE: nucleic acid
          B. LENGTH: 1186 bp
          C. STRANDEDNESS: double stranded
          D. TOPOLOGY: linear

ii)    MOLECULAR TYPE: DNA used as probe for _neo_ gene

ix)    Features:
          1 - 8 :          sequence derived from tapetum specific promoter of _Nicotiana tabacum_
          9 - 790:         coding sequence of neomycine phosphotransferase gene
          791 - end:       3' regulatory sequence containing the polyadenylation site derived from _Agrobacterium_ T-DNA gene 7

xi)    Sequence description

```
AAGCTTGGAT GGATTGCACG CAGGTTCTCC GGCCGCTTGG GTGGAGAGGC      50
TATTCGGCTA TGACTGGGCA CAACAGACAA TCGGCTGCTC TGATGCCGCC     100
GTGTTCCGGC TGTCAGCGCA GGGGCGCCCG GTTCTTTTTG TCAAGACCGA     150
CCTGTCCGGT GCCCTGAATG AACTGCAGGA CGAGGCAGCG CGGCTATCGT     200
GGCTGGCCAC GACGGGCGTT CCTTGCGCAG CTGTGCTCGA CGTTGTCACT     250
GAAGCGGGAA GGGACTGGCT GCTATTGGGC GAAGTGCCGG GGCAGGATCT     300
CCTGTCATCT CACCTTGCTC CTGCCGAGAA AGTATCCATC ATGGCTGATG     350
CAATGCGGCG GCTGCATACG CTTGATCCGG CTACCTGCCC ATTCGACCAC     400
CAAGCGAAAC ATCGCATCGA GCGAGCACGT ACTCGGATGG AAGCCGGTCT     450
TGTCGATCAG GATGATCTGG ACGAAGAGCA TCAGGGGCTC GCGCCAGCCG     500
AACTGTTCGC CAGGCTCAAG GCGCGCATGC CCGACGGCGA GGATCTCGTC     550
GTGACCCATG GCGATGCCTG CTTGCCGAAT ATCATGGTGG AAAATGGCCG     600
CTTTTCTGGA TTCATCGACT GTGGCCGGCT GGGTGTGGCG GACCGCTATC     650
AGGACATAGC GTTGGCTACC CGTGATATTG CTGAAGAGCT TGGCGGCGAA     700
TGGGCTGACC GCTTCCTCGT GCTTTACGGT ATCGCCGCTC CCGATTCGCA     750
GCGCATCGCC TTCTATCGCC TTCTTGACGA GTTCTTCTGA GCGGGACTCT     800
GGGGTTCGAA ATGACCGACC AAGCGACGCC CAACCTGCCA TCACGAGATT     850
TCGATTCCAC CGCCGCCTTC TATGAAAGGT TGGGCTTCGG AATCGTTTTC     900
CGGGACGCCG GCTGGATGAT CCTCCAGCGC GGGGATCTCA TGCTGGAGTT    950
CTTCGCCCAC CCCGATCCAT GAGCTAAGCT AGCTATATCA TCAATTTATG    1000
TATTACACAT AATATCGCAC TCAGTCTTTC ATCTACGGCA ATGTACCAGC    1050
TGATATAATC AGTTATTGAA ATATTTCTGA ATTTAAACTT GCATCAATAA    1100
ATTTATGTTT TTGCTTGGAC TATAATACCT GACTTGTTAT TTTATCAATA    1150
AATATTTAAA CTATATTTCT TTCAAGATGG GAATTC                   1186
```

4. Information for SEQ. ID. NO. 3

i)     Sequence Characteristics
        TYPE: nucleic acid
        LENGTH: 1287 bp
        STRANDEDNESS: double-stranded
        TOPOLOGY: linear

ii)    MOLECULAR TYPE: DNA comprising a chimaeric gene

ix)    FEATURES:
        1 - 545 : promoter from the TA29 gene from <u>Nicotiana tabacum</u>
        546 - 881 : coding sequence of the barnase gene
        882 - 3' regulatory sequence containing the polyadenylation
        site derived from the nopaline synthase gene from
        <u>Agrobacterium</u> T-DNA

xi)    Sequence Description:

```
ATCTAGCTAA GTATAACTGG ATAATTTGCA TTAACAGATT GAATATAGTG        50
CCAAACAAGA AGGGACAATT GACTTGTCAC TTTATGAAAG ATGATTCAAA       100
CATGATTTTT TATGTACTAA TATATACATC CTACTCGAAT TAAAGCGACA       150
TAGGCTCGAA GTATGCACAT TTAGCAATGT AAATTAAATC AGTTTTTGAA       200
TCAAGCTAAA AGCAGACTTG CATAAGGTGG GTGGCTGGAC TAGAATAAAC       250
ATCTTCTCTA GCACAGCTTC ATAATGTAAT TTCCATAACT GAAATCAGGG       300
TGAGACAAAA TTTTGGTACT TTTTCCTCAC ACTAAGTCCA TGTTTGCAAC       350
AAATTAATAC ATGAAACCTT AATGTTACCC TCAGATTAGC CTGCTACTCC       400
CCATTTTCCT CGAAATGCTC CAACAAAGT TAGTTTTGCA AGTTGTTGTG       450
TATGTCTTGT GCTCTATATA TGCCCTTGTG GTGCAAGTGT AACAGTACAA       500
CATCATCACT CAAATCAAAG TTTTTACTTA AAGAAATTAG CTACCATGGT       550
ACCGGTTATC AACACGTTTG ACGGGGTTGC GGATTATCTT CAGACATATC       600
ATAAGCTACC TGATAATTAC ATTACAAAAT CAGAAGCACA AGCCCTCGGC       650
TGGGTGGCAT CAAAAGGGAA CCTTGCAGAC GTCGCTCCGG GGAAAAGCAT       700
CGGCGGAGAC ATCTTCTCAA ACAGGGAAGG CAAACTCCCG GGCAAAAGCG       750
GACGAACATG GCGTGAAGCG GATATTAACT ATACATCAGG CTTCAGAAAT       800
TCAGACCGGA TTCTTTACTC AAGCGACTGG CTGATTTACA AAACAACGGA       850
CCATTATCAG ACCTTTACAA AAATCAGATA ACGAAAAAAA CGGCTTCCTG       900
CGGAGGCCGT TTTTTTCAGC TTTACATAAA GTGTGTAATA AATTTTTCTT       950
CAAACTCTGA TCGGTCAATT TCACTTTCCG GXXXXCTCTA GAGGATCCGA      1000
AGCAGATCGT TCAAACATTT GGCAATAAAG TTTCTTAAGA TTGAATCCTG      1050
TTGCCGGTCT TGCGATGATT ATCATATAAT TTCTGTTGAA TTACGTTAAG      1100
CATGTAATAA TTAACATGTA ATGCATGACG TTATTTATGA GATGGGTTTT      1150
TATGATTAGA GTCCCGCAAT TATACATTTA ATACGCGATA GAAAACAAAA      1200
TATAGCGCGC AAACTAGGAT AAATTATCGC GCGCGGTGTC ATCTATGTTA      1250
CTAGATCGGG AAGATCCCCG GGTACCGAGC TCGAATT                    1287
```

5. Information for SEQ. ID. NO. 4

i)      Sequence characteristics
                A. TYPE: nucleic acid
                B. LENGTH: 4883 bp
                C. STRANDEDNESS: double stranded
                D. TOPOLOGY: circular

ii)     MOLECULAR TYPE: pDE110 : plasmid DNA replicable in E. coli

ix)     Features:
                1 - 395 :           pUC18 derived sequence
                396 - 1779:         "35S3" promoter sequence derived from
                                    Cauliflower    mosaic    virus    isolate
                                    CabbB-JI
                1780 - 2331:        coding  sequence  of  phosphinotricin
                                    acetyltransferase gene
                2332 - 2619:        3' regulatory sequence containing the
                                    polyadenylation   site   derived   from
                                    Agrobacterium T-DNA nopaline synthase
                                    gene
                2620 - 4883:        pUC18 derived sequence

        Other information: plasmid is replicable in E. coli,
        confers ampicillin resistance to the bacterium

xi)     Sequence description

```
TCGCGCGTTT  CGGTGATGAC  GGTGAAAACC  TCTGACACAT  GCAGCTCCCG      50
GAGACGGTCA  CAGCTTGTCT  GTAAGCGGAT  GCCGGGAGCA  GACAAGCCCG     100
TCAGGGCGCG  TCAGCGGGTG  TTGGCGGGTG  TCGGGGCTGG  CTTAACTATG     150
CGGCATCAGA  GCAGATTGTA  CTGAGAGTGC  ACCATATGCG  GTGTGAAATA     200
CCGCACAGAT  GCGTAAGGAG  AAAATACCGC  ATCAGGCGCC  ATTCGCCATT     250
CAGGCTGCGC  AACTGTTGGG  AAGGGCGATC  GGTGCGGGCC  TCTTCGCTAT     300
TACGCCAGCT  GGCGAAAGGG  GGATGTGCTG  CAAGGCGATT  AAGTTGGGTA     350
ACGCCAGGGT  TTTCCCAGTC  ACGACGTTGT  AAAACGACGG  CCAGTGAATT     400
CCAATCCCAC  CAAAACCTGA  ACCTAGCAGT  TCAGTTGCTC  CTCTCAGAGA     450
CGAATCGGGT  ATTCAACACC  CTCATACCAA  CTACTACGTC  GTGTATAACG     500
GACCTCATGC  CGGTATATAC  GATGACTGGG  GTTGTACAAA  GGCAGCAACA     550
AACGGTGTTC  CCGGAGTTGC  GCATAAGAAG  TTTGCCACTA  TTACAGAGGC     600
AAGAGCAGCA  GCTGACGCGT  ATACAACAAG  TCAGCAAACA  GATAGGTTGA     650
ACTTCATCCC  CAAAGGAGAA  GCTCAACTCA  AGCCCAAGAG  CTTTGCGAAG     700
GCCCTAACAA  GCCCACCAAA  GCAAAAAGCC  CACTGCTCAC  GCTAGGAACC     750
AAAAGGCCCA  GCAGTGATCC  AGCCCCAAAA  GAGATCTCCT  TTGCCCCGGA     800
GATTACAATG  GACGATTTCC  TCTATCTTTA  CGATCTAGGA  AGGAAGTTCG     850
AAGGTGAAGG  TGACGACACT  ATGTTCACCA  CTGATAATGA  GAAGGTTAGC     900
CTCTTCAATT  TCAGAAAGAA  TGCTGACCCA  CAGATGGTTA  GAGAGGCCTA     950
CGCAGCAGGT  CTCATCAAGA  CGATCTACCC  GAGTAACAAT  CTCCAGGAGA    1000
TCAAATACCT  TCCCAAGAAG  GTTAAAGATG  CAGTCAAAAG  ATTCAGGACT    1050
AATTGCATCA  AGAACACAGA  GAAAGACATA  TTTCTCAAGA  TCAGAAGTAC    1100
TATTCCAGTA  TGGACGATTC  AAGGCTTGCT  TCATAAACCA  AGGCAAGTAA    1150
TAGAGATTGG  AGTCTCTAAA  AAGGTAGTTC  CTACTGAATC  TAAGGCCATG    1200
CATGGAGTCT  AAGATTCAAA  TCGAGGATCT  AACAGAACTC  GCCGTGAAGA    1250
CTGGCGAACA  GTTCATACAG  AGTCTTTTAC  GACTCAATGA  CAAGAAGAAA    1300
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ATCTTCGTCA ACATGGTGGA GCACGACACT CTGGTCTACT CCAAAAATGT    1350
CAAAGATACA GTCTCAGAAG ACCAAAGGGC TATTGAGACT TTTCAACAAA    1400
GGATAATTTC GGGAAACCTC CTCGGATTCC ATTGCCCAGC TATCTGTCAC    1450
TTCATCGAAA GGACAGTAGA AAAGGAAGGT GGCTCCTACA AATGCCATCA    1500
TTGCGATAAA GGAAAGGCTA TCATTCAAGA TGCCTCTGCC GACAGTGGTC    1550
CCAAAGATGG ACCCCCACCC ACGAGGAGCA TCGTGGAAAA AGAAGACGTT    1600
CCAACCACGT CTTCAAAGCA AGTGGATTGA TGTGACATCT CCACTGACGT    1650
AAGGGATGAC GCACAATCCC ACTATCCTTC GCAAGACCCT TCCTCTATAT    1700
AAGGAAGTTC ATTTCATTTG GAGAGGACAC GCTGAAATCA CCAGTCTCTC    1750
TCTATAAATC TATCTCTCTC TCTATAACCA TGGACCCAGA ACGACGCCCG    1800
GCCGACATCC GCCGTGCCAC CGAGGCGGAC ATGCCGGCGG TCTGCACCAT    1850
CGTCAACCAC TACATCGAGA CAAGCACGGT CAACTTCCGT ACCGAGCCGC    1900
AGGAACCGCA GGAGTGGACG GACGACCTCG TCCGTCTGCG GGAGCGCTAT    1950
CCCTGGCTCG TCGCCGAGGT GGACGGCGAG GTCGCCGGCA TCGCCTACGC    2000
GGGCCCCTGG AAGGCACGCA ACGCCTACGA CTGGACGGCC GAGTCGACCG    2050
TGTACGTCTC CCCCCGCCAC CAGCGGACGG GACTGGGCTC CACGCTCTAC    2100
ACCCACCTGC TGAAGTCCCT GGAGGCACAG GGCTTCAAGA GCGTGGTCGC    2150
TGTCATCGGG CTGCCCAACG ACCCGAGCGT GCGCATGCAC GAGGCGCTCG    2200
GATATGCCCC CCGCGGCATG CTGCGGGCGG CCGGCTTCAA GCACGGGAAC    2250
TGGCATGACG TGGGTTTCTG GCAGCTGGAC TTCAGCCTGC CGGTACCGCC    2300
CCGTCCGGTC CTGCCCGTCA CCGAGATCTG ATCTCACGCG TCTAGGATCC    2350
GAAGCAGATC GTTCAAACAT TTGGCAATAA AGTTTCTTAA GATTGAATCC    2400
TGTTGCCGGT CTTGCGATGA TTATCATATA ATTTCTGTTG AATTACGTTA    2450
AGCATGTAAT AATTAACATG TAATGCATGA CGTTATTTAT GAGATGGGTT    2500
TTTATGATTA GAGTCCCGCA ATTATACATT TAATACGCGA TAGAAAACAA    2550
AATATAGCGC GCAAACTAGG ATAAATTATC GCGCGCGGTG TCATCTATGT    2600
TACTAGATCG GGAAGATCCT CTAGAGTCGA CCTGCAGGCA TGCAAGCTTG    2650
GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC    2700
AATTCCACAC AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG    2750
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AACCAGCCAG CCGGAAGGGC CGAGCGCAGA AGTGGTCCTG CAACTTTATC    4050
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## Claims

1. A method for transforming, with a DNA, a genome, particularly a nuclear genome, of a monocotyledonous plant, especially a gramineous plant, particularly a cereal plant such as corn, wheat, rice, oats, barley, sorghum, rye and millet, quite particularly corn, comprising the steps of:

    a) wounding and/or degrading either an intact tissue of said plant that is capable of forming compact embryogenic callus or a compact embryogenic callus, particularly an embryogenic sector thereof, obtained from said intact tissue of said plant, so as to render a cell of said intact tissue or callus competent with respect to i) uptake of said DNA ii) integrative transformation of said DNA in said plant genome and 3) regeneration of said plant from said cell;

    b) transforming said competent cell with said DNA; and then

    c) regenerating, from said transformed competent cell, said plant as a phenotypically normal plant, such as a phenotypically normal, fertile mature plant.

2. The method of claim 1 wherein said intact tissue is wounded by being cut to a maximum dimension of 0.1 to 5 mm, preferably 1 to 2.5 mm, particularly 1.25 to 1.75 mm, and then said competent cell so-produced is transformed.

3. The method of claim 1 or 2 wherein said intact tissue is wounded by being cut and then is degraded by being treated with an enzyme, preferably to generate pores in cell walls of said intact tissue, and then said competent cell so-produced is transformed.

4. The method of claim 1 wherein said plant is corn, an intact immature corn embryo is degraded by being treated with an enzyme, preferably to generate pores in cell walls of said embryo, and then said competent cell so-produced is transformed.

5. The method of claim 1, wherein said callus is wounded by being cut to a maximum length of 0.5 to 2.5 mm, particularly 1 to 2 mm, quite particularly 1.25 to 1.75 mm, and preferably to a minimum length of about 0.1 mm, and then said competent cell so-produced is transformed.

6. The method of claim 1 or 5 where said callus is wounded by being cut and then is degraded by being treated with an enzyme, preferably to generate pores in cell walls of said callus, and then said competent cell so-produced is transformed.

7. The method of claim 1 wherein said callus is degraded by being treated with an enzyme to generate pores in cell walls of said callus, and then said competent cell so-produced is transformed.

8. The method of any one of claims 1-7 wherein said competent cell is transformed by direct gene transfer, particularly by electroporation.

9. A gramineous plant, especially a cereal, particularly corn or rice, having its genome, particularly its nuclear genome, integratively transformed with a DNA, particularly a chimaeric gene, expressible in said plant; said plant being characterized by the fact that, under conventional culture conditions such as are described by Datta et al (1990) Bio/Technology 8:736, Shimamoto et al (1989) Nature 338:274, Gordon-Kamm et al (1990) The Plant Cell 2:603, and Fromm et al (1990) Bio/Technology 8:833, it is practically impossible to obtain an embryogenic suspension culture or protoplasts of cells of said

plant that are competent with respect to 1) uptake of said DNA, 2) integrative transformation of said DNA into said plant genome and 3) regeneration of said plant as a phenotypically normal, fertile plant transformed with said DNA.

10. The plant of claim 9, that is of a line, such that under said culture conditions, it is practically impossible to regenerate the plant as a phenotypical normal plant, such as a phenotypically normal, fertile plant, of said line from a transformed embryogenic suspension culture or transformed protoplasts of said line, particularly where for every 10,000 untransformed protoplasts of said line, no more than about 500, especially no more than about 100, particularly no more than about 10, quite particularly no more than about 1, phenotypically normal plant(s) can be regenerated.

11. A transformed plant, especially a moncot, particularly a gramineous plant, quite particularly a cereal such as corn or rice, made by the method of any one of claims 1-8.

12. A corn plant of any one of claims 9-11 which has the capacity to form type I callus but does not have the capacity to form type II callus, under any of the culture conditions described by Gordon-Kamm et al (1990) The Plant Cell 2:603 and Fromm et al (1990) Bio/Technology 8:833, at frequences higher than 10%, particularly at frequencies higher than 1%, quite particularly at frequencies higher than 0.1%, more quite particularly at frequencies higher than 0.01%.

13. A cell of the plant of any one of claims 9-12 integratively transformed with said DNA or a culture consisting essentially of a plurality of said cells.

14. A seed of the transformed plant of any one of claims 9-12.

15. A plant of any one of claims 9-12 which is a pollinator plant, such as for the production of hybrid plants.

16. A hybrid resulting from the crossing of the pollinator plant of claim 15 and another inbred line of plant.

17. A male-sterile monocot plant, preferably a gramineous plant, particularly a corn plant, quite particularly the corn plant of claim 12, or a seed of the male-sterile plant, transformed with a coding sequence that encodes a protein capable of killing or disabling plant cells in which the protein is expressed and that is under the control of the tapetum-specific PTA29 promoter.

18. A cell of the transformed monocot plant of claim 17 or a culture consisting essentially of a plurality of said cells.
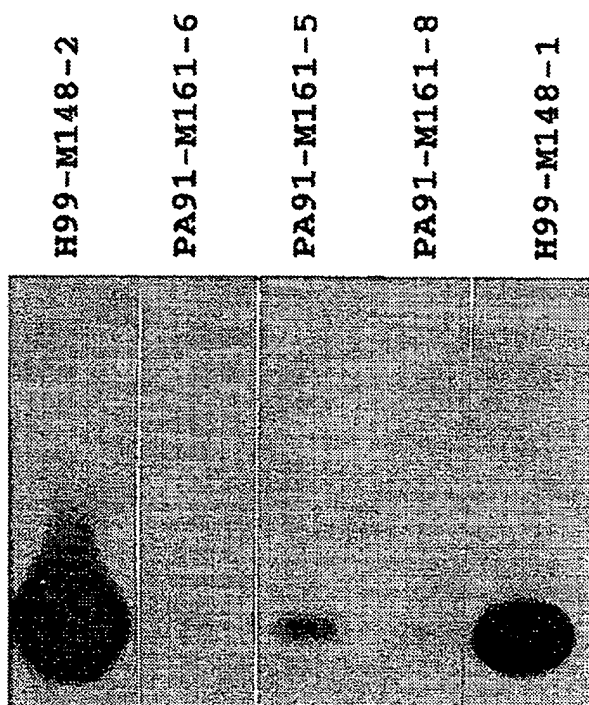
FIG. 1

# FIG. 2

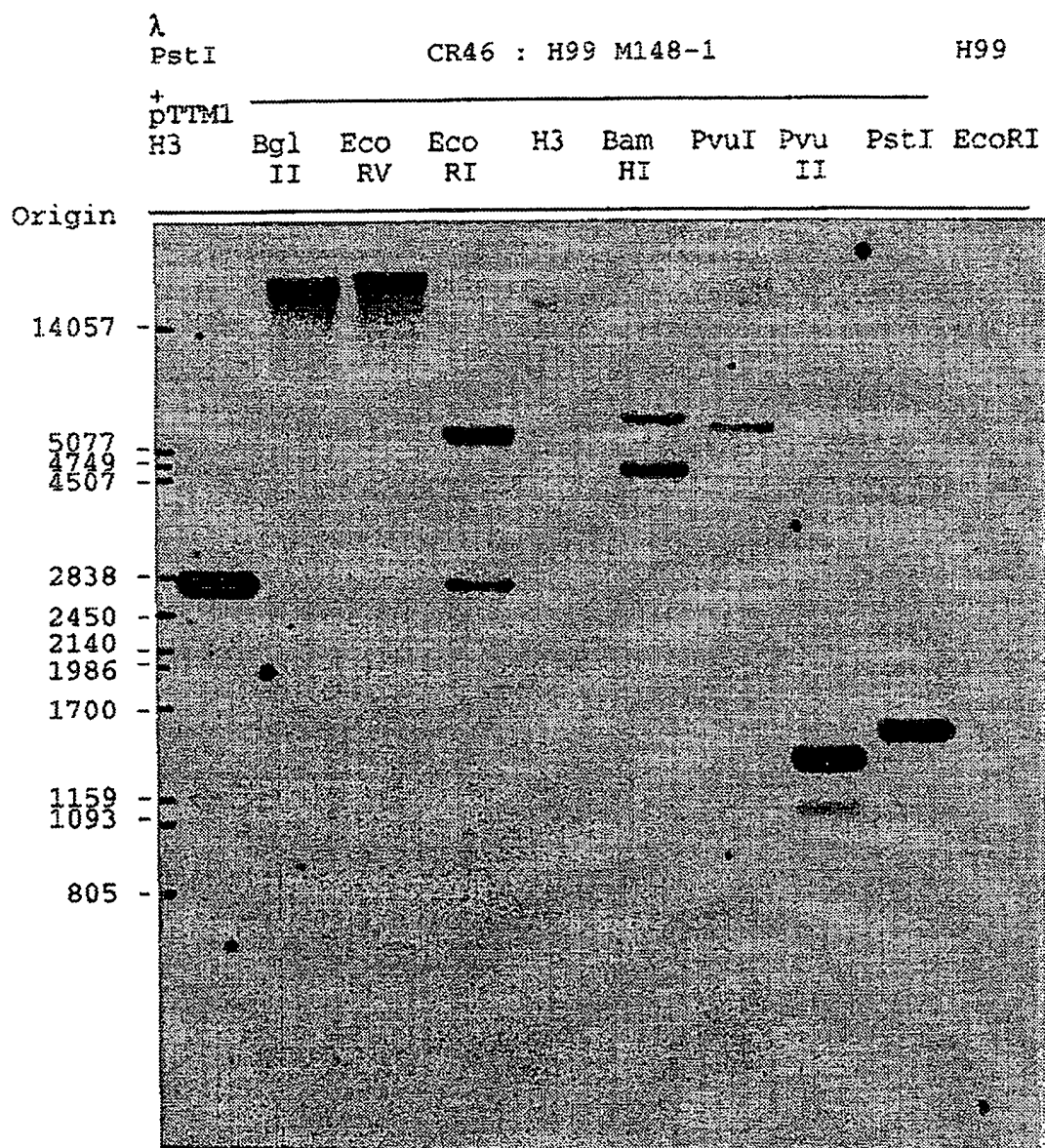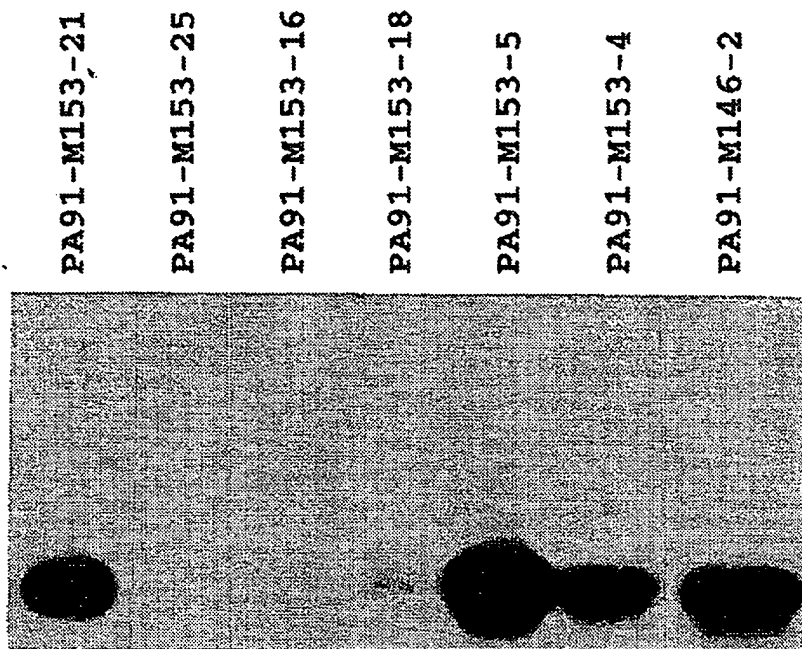Blot : Corn 7    Probe pTTM1 E1-H3 for NPTII
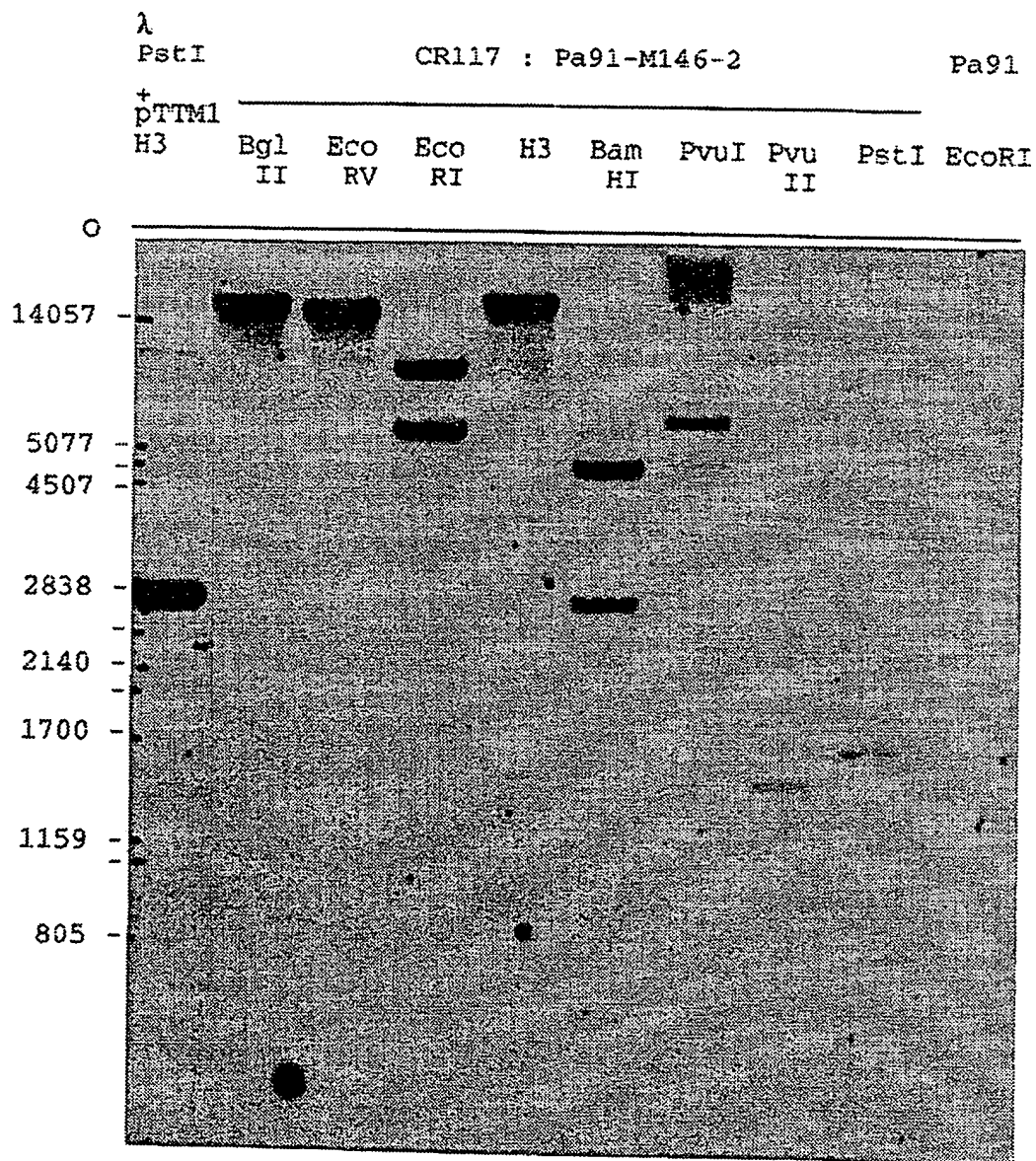
Exp : 48 hours

FIG. 3

# FIG. 4

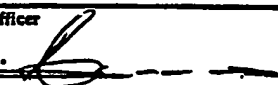Blot : Corn 8    Probe pTTM1 E1-H3 for NPTII

Exp : 48 hours

| I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6] |
|---|

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82;          A01H5/00;          C12N5/10

| II. FIELDS SEARCHED |
|---|

| Minimum Documentation Searched[7] | |
|---|---|
| Classification System | Classification Symbols |
| Int.Cl. 5 | C12N ;      A01H |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched[8]

| III. DOCUMENTS CONSIDERED TO BE RELEVANT[9] | | |
|---|---|---|
| Category[°] | Citation of Document,[11] with indication, where appropriate, of the relevant passages[12] | Relevant to Claim No.[13] |
| X | NL,A,8 801 444 (SOLVAY) 2 January 1990<br><br>see page 6, line 24 – page 7, line 20<br>see page 7, line 36 – page 12, line 16<br>--- | 1,3,4,<br>8-16 |
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| | -/-- | |

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

| IV. CERTIFICATION | |
|---|---|
| Date of the Actual Completion of the International Search<br><br>18 FEBRUARY 1992 | Date of Mailing of this International Search Report<br><br>16.03.92 |
| International Searching Authority<br><br>EUROPEAN PATENT OFFICE | Signature of Authorized Officer<br><br>MADDOX A.D. |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT | (CONTINUED FROM THE SECOND SHEET) | |
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| P,X | DE,A,4 013 099 (HOECHST) 31 October 1991 see page 4, line 19 - line 29 | 1,5,8-16 |
| A | THE PLANT CELL. vol. 2, no. 7, July 1990, ROCKVILLE, MD, USA. pages 591 - 602; DEKEYSER, R. A., ET AL.: 'Transient gene expression in intact and organized rice tissues' see page 660, left column, paragraph 2 | 1-16 |
| A | EP,A,O 203 790 (UNIVERSITY OF NOTTINGHAM) 3 December 1986 see claims 1-10 | 1-16 |
| A | DE,A,3 738 874 (INSTITUT BOTANIKI UKRAINSKOJ) 17 November 1988 see example 5 | 1-16 |

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The members are as contained in the European Patent Office EDP file on
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